PHARMACOGNOSTICAL, PHYTOCHEMICAL AND HYPOLIPIDEMIC ACTIVITY ON THE LEAVES OF *Operculina turpethum* (L.) Silva Manso

A dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI - 600 032

In partial fulfilment of the requirements for the award of degree of

MASTER OF PHARMACY
IN
PHARMACOGNOSY

Submitted by
Reg.No. 261220658

Under the guidance of

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This is to certify that the dissertation entitled "**PHARMACOGNOSTICAL, PHYTOCHEMICAL AND HYPOLIPIDEMIC ACTIVITY ON THE LEAVES OF *Operculina turpethum* silva manso,**" submitted by **Reg. No. 261220658,** in partial fulfilment of the requirements for the award of the degree of **Master of Pharmacy in Pharmacognosy** by The Tamil Nadu Dr. M.G.R. Medical University, Chennai, is a bonafide record of work done by her during the academic year 2013 – 2014 under the guidance of **Dr. N.Jayshree, M.Pharm., Ph.D.,** Professor & Head, Department of Pharmacognosy, Madras Medical College, Chennai – 600 003.

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Dr. N. JAYSHREE

Place: Chennai-03.

Date:

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To Mom and Dad,
who always picked me up on time
and encouraged me to go on every adventure,
especially this one

DEDICATED TO MY PARENTS

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Authentication Certificate

Certified that the plant material submitted by **S.Zaburuth Nisha** wasCarefully Examined and botanically identified as **Operculina turpethum(Linn.)Silva Manso** Of the family **Convolvulaceae.**

Date: 16.9.2013

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TITLE:

"PHARMACOGNOSTICAL, PHYTOCHEMICAL AND HYPOLIPIDEMIC ACTIVITY ON THE LEAVES OF *Operculina turpethum*"

The Animal Ethical Clearance Committee experts screened her proposal Vide 7/243/CPCSEA and have given clearance in the meeting held on 22.11.13 at Dean's Chamber in Madras Medical College.

17.2.14

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

Cardiovascular disease[1,2]

Cardiovascular disease (CVD), particularly coronary heart disease (CHD) is the leading cause of medically certified death in both developed as well as developing countries. It is reported that almost 12 million people die of CHD disease each year all over the world. The average onset of CHD is younger among Indians than in other populations around the world.

CHD is a disease in which a waxy substance called plaque builds up inside the coronary arteries. Normally these arteries supply oxygen-rich blood to heart muscle. The build up of plaque, limits the flow of oxygen-rich blood through the artery and leads to Angina, Heart attack etc.,

Causes[3]

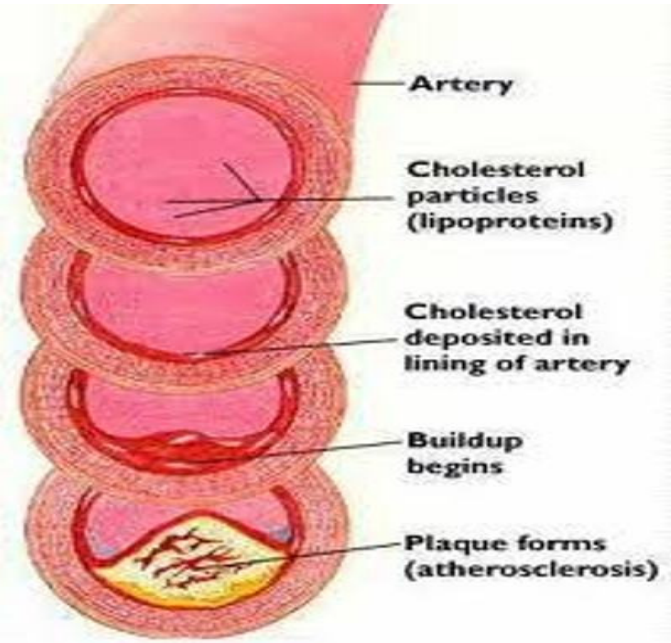
- Unhealthy diet
- Physical inactivity
- Atherosclerosis
- Tobacco use
- Harmful use of alcohol

Atherosclerosis in relation to CHD[4]

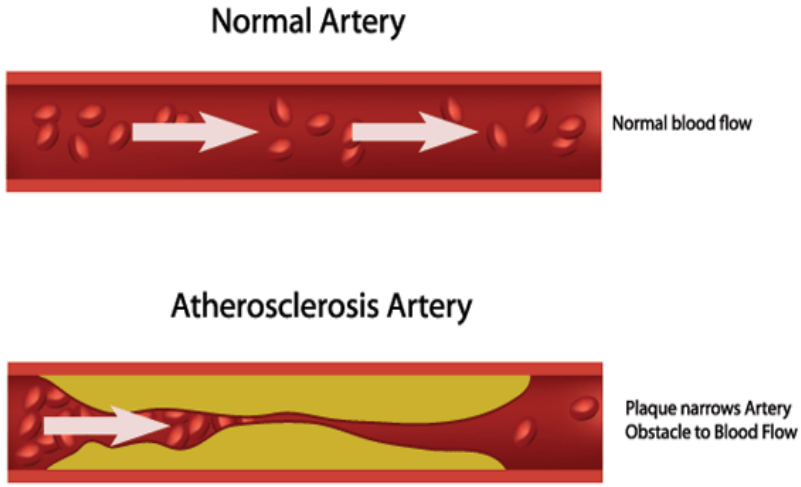
Atherosclerosis was found to be one of the major causes for this disease. It is estimated that by the year 2020, CVD, mainly due to atherosclerosis, will become the leading cause of total global disease burden.

Atherosclerosis[5- 7]

High lipid levels can speed up a process called **Atherosclerosis** or hardening of arteries. It referred to as a **"silent killer"**. The term atherosclerosis is derived from **Athero-** referring to the soft lipid rich material in the centre of atheroma and **sclerosis-** connective tissue in the plaques. Arteries are normally smooth and unobstructed inside, but as the lipid level increases, a sticky substance called plaque forms in the wall of arteries.



Plaque is made of lipids and other materials circulating in the blood. As more plaque builds up, arteries become narrow and stiff. Eventually, enough plaque may build up to reduce blood flow through the arteries.



Atherosclerotic lesions are composed of three major components

- ❖ Cellular component comprised predominantly of smooth muscle cells and macrophages
- ❖ Connective tissue matrix and extracellular lipid
- ❖ Intracellular lipid that accumulates within macrophages, thereby converting them into foam cells.

Table 1: Risk factors in Atherosclerosis

Major risk factors	**Emerging risk factors**
a) Modifiable	**Environmental influences**
➢ **Dyslipidemia**	**Obesity**
➢ **Hypertension**	**Hormone deficiency**
➢ **Diabetes mellitus**	**Physical inactivity**
➢ **Smoking**	**Stressful life**
b) Constitutional	**Homocystinuria**
➢ **Age**	**Role of alcohol**
➢ **Sex**	**Prothrombotic factors**
➢ **Genetic factors**	**Infections (C. Pneumonia, etc,.)**

Lipid[8,9]

It is the scientific term for fats in the blood. Since blood and other body fluids are watery, fats needs a special transport system to travel around the body. They are carried from one place to another by mixing with protein particles, called Lipoproteins. Lipoproteins are divided into five major classes according to density such as

- **Chylomicrons**

  Chylomicrons are made by the intestine for carrying new fat to the cells of the body and they carry mostly triglycerides.

- **Very Low Density Lipoproteins (VLDL)**

  VLDL is made by the liver and intestine to carry fats around the body and they carry mostly triglycerides.

- **Intermediate Density Lipoprotein (IDL)**

  IDL refers to a density between HDL & LDL and also between LDL & VLDL. It transports a variety of triglyceride fats, cholesterol and promotes the growth of atheroma.

- **Low Density Lipoproteins (LDL)**

  LDL is made by the liver to transport cholesterol to the body's cells and tissues. It may form deposits on the walls of the arteries and other blood vessels. They are therefore called **"Lazy or Bad cholesterol".**

- **High Density Lipoproteins (HDL)**

  HDL picks up and transport excess cholesterol from the walls of arteries and brings it back to the liver for processing and removal. They are therefore called **"Healthy or Good cholesterol".**

Table 2: Normal levels of lipids

Types	**Desirable mg/dL**
Cholestrol	<200
LDL Cholestrol	<130
HDLCholestrol	≥60
Triglyceride	<200

At normal levels, lipids perform important function in the body but, can cause health problems if they are present in excess. This condition is termed as hyperlipidemia.

Hyperlipidemia[10,11]

Hyperlipidemia is a condition characterized by elevation of one or more lipids including cholesterol, cholesterol esters, phospholipids and triglycerides in the blood stream. It is also called as hyperlipoproteinemia, because these fatty substances travel in the blood attached to proteins.

Table 3: Normal and Abnormal levels of lipids

Types	**Desirable mg/dL**	**Borderline high risk mg/dL**	**High risk for CHD mg/dL**
Cholestrol	<200	200-239	≥240
LDL Cholestrol	<130	130-159	≥160
HDLCholestrol	≥60	-	□35
Triglyceride	<200	200-400	400-1000

Genetic predisposition and secondary causes like underlying disease states, medications and life-style are some of the causes for hyperlipidemia. The severe forms of hyperlipidemia occur in those with inherited traits which results in defects in the lipid metabolism. Diet and life-style shows a significant effect on lipid profiles. Body weight is positively correlated with LDL cholesterol and triglycerides, and negatively correlated with HDL cholesterol. Dietary intake is one of the key factors in determining the lipid profile, which led to the dietary changes in patients with hyperlipidemia. Hypothyroidism is also the most common cause of secondary hyperlipidemia.

The increased serum cholesterol levels have been directly linked to the risk of CHD, whereas LDL cholesterol is more closely associated with risk and extent of this disease.

Currently available drugs for the treatment of hyperlipidemia[12]

- HMG CoA reductase (statins)

  Lovastatin, Atorvastatin, Pravastatin, Simvastatin, Rosuvastatin

- Bile acid sequestrants (resins)

  Cholestyramine, Colestipol

- Activate lipoprotein lipase (fibric acid derivatives)

  Clofibrate, Gemfibrozil, Benzafibrate, Fenofibrate

- Inhibit lipopysis and triglyceride synthesis

  Nicotinic acid.

Disadvantages of allopathic drugs[13]

Currently available allopathic drugs have been associated with number of side effects. The consumption of synthetic drugs leads to epigastric pain, hyperuricemia, abdominal cramps, diarrhoea, nausea, myositis, flatulence, leucopenia, flushing, dry skin and abnormal liver function.

Herbal medicine and its popularity[14-16]

Herbal medicine is still the mainstay of about 75–85% of the world population, mainly in the developing countries, for Primary Health Care. This is primarily because of the general belief that herbal drugs are without any side effects besides being cheap & locally available.

India is one of the 12 mega biodiversity centres having over 45,000 plant species. About 1500 plants with medicinal uses are mentioned in ancient texts & around 800 plants have been used in the traditional medicine.

India has a rich tradition of herbal medicine as evident from Ayurveda, which could not have flourished for two thousand years without any scientific basis.

Hyperlipidemia – Herbal review[17,18]

Medicinal plants are also known to play a major role in treatment of hyperlipidemia. A large number of plants have been traditionally used in the treatment of hyperlipidemia. Literature survey suggests that the lipid lowering action is mediated through inhibition of hepatic cholesterol biosynthesis and reduction of lipid absorption in the intestine.

Some of the common herbs with Hypolipidemic activity are

Achyranthes aspera, Anacardium occidentale, Apium graveolans, Centella asiatica, Commiphora mukul, Cucurbita maxima, Curcuma longa, Cynara scolymus, Ganoderma lucidum, Ginko biloba, Medicago sativa, Momordica charantia, Musa sapiens, Nigella sativa, Phyllanthus niruri, Plantago psyllium, Polygonum multiflorum, Rheum palmatum, Oenothera biennis, Silybum marianum, Terminalia arjuna.

The present work is an attempt to determine the antihyperlipidemic activity of *Operculina turpethum*. This plant has a folklore claim in the treatment of hyperlipidemia but no studies have been reported so far.

2. RATIONALE OF THE STUDY

- Hyperlipidemia is one of the greatest risk factors in the manifestation and development of atherosclerosis and cardiac heart disease (CHD). CHD is the leading cause of death in both developed as well as developing countries. It affects Indians with greater frequency at younger age. It is expected that there would be around 62 million patients with CHD by 2015 in India.
- The available allopathic drugs though effective, have several side effects. This had created a vital necessity for finding natural therapy for the treatment of hyperlipidemia.
- Medicinal plants have been shown to play a major role in treating hyperlipidemia. Recently herbal hypolipidemics have gained importance to fill the lacunae created by synthetic drugs.
- *Operculina turpethum* is one such plant with promising traditional and folklore claims. It is commonly known as **Indian jalap** or **Transparent wood rose**. It is useful in treating ulcer, diabetes, hyperlipidemia, cancer, constipation and liver disorders.
- Traditional use of hypolipidemic activity has not yet been confirmed scientifically. Hence the present study attempts to study the pharmacognostical, phytochemical and hypolipidemic activity on the leaves of *Operculina turpethum.*

3. REVIEW OF LITERATURE

The literature survey of the plant *Operculina turpethum*(L). Silva Manso reveals the following

- Ignatius V *et al.* (2013) reported the anti-ulcer activity of methanolic stem extract of *Operculina turpethum.* They have concluded that the methanolic extract of *Operculina turpethum* healed the penetrating ulcer induced by aspirin after 10 days treatment at a dose of 200mg/kg with a percentage inhibition of 60.13% and ulcer index of 1.7 when compared to standard ranitidine which has 65.14% inhibition and an ulcer index of 1.5 [19].
- Sharma *et al.* (2013) reported comparative analysis of phytochemicals such as alkaloids, glycosides, tannins, saponins, phenols, flavonoids, sugars, terpenoids and steroids present in different extracts of *Operculina turpethum* root [20].
- Shankaraiah *et al.* (2012) evaluated methanolic extract of *Operculina turpethum* and showed its anti-diabetic activity against healthy and Streptozotocin induced diabetes in rats. In their study they have stated that methanolic extract of *Operculina turpethum* supplementation is quite beneficial in controlling the blood glucose level[21].
- Neuropharmacological effects of aqueous and ethanolic extract of root of *Operculina turpethum* in Swiss albino mice was reported by Prathyusha *et al.* (2012). They have concluded that both the extracts exhibited better motor coordination in comparison with standard diclofenac [22].
- Veena S *et al.* (2012) reported *in vitro* radical scavenging activity of ethanolic extract of *Operculina turpethum,* which may be attributed to the presence of substantial amount of phenolic and flavonoid content in the extract [23].
- Isolation of four new dammarane type triterpenoids from aerial parts of *Operculina turpethum* was carried out by Ding W *et al.* (2012) and the structure was elucidated by spectroscopic analysis and chemical correlation. Out of these, two compounds showed significant protective against D-galactosamine induced toxicity in L-02 human cells [24].

- Analgesic activity of different solvent extracts of *Operculina turpethum* was carried out by Prabhavathi N *et al.* (2012) by using tail immersion method in Swiss albino mice. They have concluded that chloroform extract showed significant activity with % inhibition at 70.5 compared to standard diclofenac sodium at 76% [25].
- Pulipaka *et al.* (2012) reported pharmacognostical studies of the root of *Operculina turpethum* (L.) Silva manso. They have reported the character of roots such as prism type of calcium oxalate crystals and simple starch grains [26].
- Jahangir M *et al.* (2010) studied the anti-microbial activity on leaves of *Operculina turpethum* and have proved that ethanolic extracts showed a varied degree of inhibition against pathogenic bacteria than petroleum ether and aqueous extract. They also have reported that the zone of inhibition of ethanolic extract ranged from 9 to 14mm in diameter at a concentration of 5.0 mg / ml [27].
- Gopi Sudheer *et al.* (2010) reported that the alcoholic extract of *Operculina turpethum* showed hepatoprotective and antioxidant activity against anti-TB drugs induced hepatotoxicity in rats and have reported that at the maximum dose of 400mg/kg hepatotoxicity against isoniazid and rifampicin induced group was prevented. The extract also showed antioxidant activity due to the presence of sitisterol and lupeol [28].
- Ahmad R *et al.* (2009) evaluated therapeutic effects of root of *Operculina turpethum* against NDMA- induced hepatotoxicity and clastogenicity in rats and have concluded that administration of *Operculina turpethum* showed absence of fibrosis in liver and induction of micronuclei in bone marrow cells [29].
- Ashok Kumar *et al.* (2009) reported histological and physico-chemical evaluation of root of *Operculina turpethum* Linn [30].
- Anbuselvam C *et al*. (2007) studied protective effect of *Operculina turpethum* against 7,12 DMBA induced oxidative stress with reference to breast cancer in rats and have proved that the methanolic extract of stem of *Operculina turpethum* at a dose of 100mg/kg showed significant decrease in breast tumour weight ($P<0.05$) [31].
- Sharma *et al.* (2007) reported that an Ayurvedic herbal formulation named Chaitas Ghirta containing *Operculina turpethum* along with ten other root mixtures was useful in improving major symptoms of depression in patients [32].

- Rajashekar M *et al.* (2006) investigated a formulation of *Operculina turpethum* for anti-ulcer, anti-inflammatory and anti-diarrheal properties by using Shay rats. The results revealed that the formulations have reduced the hyperacidity to the extent of 50-55% at 100mg/kg dose level and have reduced the charcoal movement significantly in charcoal meal test in mice at 400mg/kg dose. It also has reduced the paw edema volume in formalin induced inflammation rats at 100mg/kg [33].
- Suresh Kumar S.V *et al.* (2006) reported Hepatoprotective activity of ethanolic extract of root against paracetamol induced hepatic damage in rats. In their study they have concluded that *Operculina* extract showed preventive effect against paracetamol induced hepatocellular damage in rats [34].
- Harun *et al.* (2002) investigated the antibacterial and cytotoxic activities of different extracts and isolated compound of *Operculina turpethum* using brine shrimp lethality assay models and have reported that for brine shrimp assay, ethyl acetate extract showed significant antibacterial and less potent than that of standard kanamycin. They also have reported that the extract obtained may contain safe and effective chemotherapeutic agent [35].
- Khare AK *et al.* (1982) reported that ethanolic and aqueous extract of *Operculina turpethum* root showed significant anti-inflammatory against carragenan induced rat paw edema as well as cotton pallet induced granuloma [36].

4. ETHNOBOTANICAL SURVEY [37, 38]

Fig 1: *Operculina turpethum* (L.) Silva Manso

Plant introduction

Biological name	:	*Operculina turpethum*
Synonyms	:	*Ipomoea turpethum* R. Br
		Merremia turpethum Linn
Family	:	Convolvulaceae

Vernacular names

English	:	Indian jalap
Tamil	:	Kumbam, Shivadai
Sanskrit	:	Triputa, Kalaparni
Hindi	:	Nisotar, Nisoth
Telugu	:	Telletegada, Tegada
Malayalam	:	Rochani, Chivaka
Kannada	:	Bangada balli

Taxonomical classification

Kingdom	:	Plantae
Subkingdom	:	Viridaeplantae
Division	:	Tracheophyta
Subdivision	:	Spermatophytina
Class	:	Magnoliopsida
Super order	:	Asternae
Order	:	Solanales
Family	:	Convolvulaceae
Genus	:	*Operculina*
Species	:	*turpethum*

Geographical distribution

Operculina turpethum is native to Africa, Asia Temperate and Tropical and Australia while it is naturalised in West Indies. It is abundantly found in Srilanka, China, Australia, Pacific islands and India.

In India, it is found in local areas of Tamil Nadu, Andhra Pradesh and Karnataka.

Habit

It is a large, stout, perennial climber, grows up to a height of 1000 m.

It is occasionally grown in gardens as an ornamental plant.

Morphology [39, 40]

Leaves are simple, large and much variable in shape such as ovate, petiolate, subacute, mucronate and more or less pubescent on both sides, base cordate or truncate. Base of the leaf is 5-10 cm long and 1.3-7 cm wide.

Flowers are white, campanulate with sepals 4-5 cm long, funnel shaped. In few flowered cymes with globose capsules enclosed within overlapping brittle sepals.

Fruits are capsules, roundish, globose with 4 seeds, which are glabrous and dull black. The plant bears fruits and flowers in March and December.

Roots are 1.5-15 cm long and 1-5 cm in diameter, usually unbranched, cylindrical, elongated, bearing thin rootlets, thicker, occasionally split and show central wood portions.

Stems are long, twisting, pubescent with angled, winged which become very tough and brown when old.

Parts used

Leaves, stems and roots.

Chemical constituents [41]

The plant contains a wide variety of phytoconstituents such as turpethin, turpethin α and β, glycosidic resin, coumarins, β sitosterol, scopoletin and essential oils.

Ethnobotanical uses [42]

Roots – Used to treat fevers, anoerexia, oedema, anaemia, constipation and other skin disorders.

Leaves – Fresh juice of the leaves is dropped into eyes to treat diseases like corneal opacity and conjunctivitis.

In Siddha system, it is used for warm infestation and rat bite poisoning.

Other uses

- ✓ Ulcer
- ✓ Bronchitis
- ✓ Diabetes
- ✓ Hyperlipidemia
- ✓ Cancer
- ✓ Liver disorders.

5. AIM AND OBJECTIVE

The aim of the present study was

- To standardize the leaves of *Operculina turpethum* by carrying out pharmacognostical, physico-chemical and phytochemical studies.
- To evaluate the antioxidant activity of the various extracts of leaves of *Operculina turpethum*.
- To evaluate the *in vitro* hypolipidemic activity of the various extracts of *Operculina turpethum* by inhibition of chicken pancreatic lipase model.
- Evaluation of *in vivo* hypolipidemic activity by diet induced hyperlipidemia model in rats.
- Isolation of active constituents by column chromatography.

6. PLAN OF WORK

I. PHARMACOGNOSTICAL STUDIES

- Macroscopical studies
- Microscopical studies
- Powder microscopy
- Quantitative microscopy – Leaf constants
- Linear measurements
- Determination of Physico-chemical constants
    - Ash values
        - Total ash value
        - Acid insoluble ash value
        - Water soluble ash value
        - Sulphated ash value
    - Extractive value
        - Water soluble extractive value
        - Alcohol soluble extractive value
        - Non volatile ether soluble extractive value
        - Volatile ether soluble extractive value
    - Determination of Loss on Drying
    - Determination of Foaming Index
    - Determination of Swelling Index
- Qualitative and Quantitative estimation of Heavy metals and Inorganic elements

II. PHYTOCHEMICAL STUDIES

- Preparation of Extracts
- Preliminary Phytochemical Screening of Powder and Extracts
- Quantitative estimation of Phytoconstituents
- Fluorescence Analysis of Plant Powder and Extracts

III. SELECTION OF ACTIVE EXTRACT BY *IN-VITRO* STUDIES

- *In-vitro* Chicken Pancreatic Lipase inhibition potential test
- Anti-oxidant activity

IV. CHROMATOGRAPHIC PROFILE OF ETHANOLIC EXTRACT

- TLC

V. ISOLATION AND CHARACTERISATION OF PHYTOCONSTITUENTS

- Column Chromatography
- Thin Layer Chromatography of Isolated Fractions

VI. SPECTRAL STUDIES

- Interpretation of UV Spectroscopy
- Interpretation of IR Spectroscopy
- Interpretation of NMR Spectroscopy
- Interpretation of Mass Spectroscopy
- Structural elucidation of isolated compound

VII. PHARMACOLOGICAL STUDIES

- Acute toxicity testing
- Hypolipidemic activity
    - Body weight
    - Biochemical parameters
    - Atherogenic index
    - Histopathological studies on Aorta.

7. METHODOLOGY

I. PHARMACOGNOSTICAL STUDIES [43]

Evaluation of a drug means confirmation of its identity, determination of its quality purity, and detection of nature of adulteration .Quality control of crude can be attempted by different methods of evaluation depending upon the morphological and microscopical studies of the crude drugs or their physical, chemical and biological behaviour. Systematic identification of crude drugs and their quality assurance forms an integral part of drug description.

Pharmacognostical studies include identification, authentication and standardization through morphological, microscopical, physico-chemical observations as per WHO guidelines. In herbals and herbal drugs, standardization starts with correct identity of sample, organoleptic evaluation, pharmacological evaluation, volatile matter, quantitative evaluation, phytochemical evaluation, toxicity testing and biological activity.

It has a special significance as it is directly linked with the activity of the herbal drugs.

The pharmacognostical studies for all other parts of the plant have already been reported except leaves. Hence the present study was carried out to establish the pharmacognostical features of leaves.

MATERIALS AND METHODS

- **Collection of specimen**

The leaves of the plant of *Operculina turpethum*(Linn.) Silva Manso were collected from Mekkarai, Tenkasi, Tirunelveli Dist, Tamilnadu, during September 2013. The collected plant material was authenticated by Botanist Dr. V. Chelladurai, Research officer-Botany, Central Council for Research in Ayurveda and Siddha, Tirunelveli. The leaves were shade dried, coarsely powdered and used for further studies.

MACROSCOPY

Medicinal plant materials are categorized according to sensory, macroscopic and microscopic characteristics. Organoleptic evaluation provides the simplest and quickest means to establish the identity, purity and quality of a particular sample. Hence, this observation is of primary importance before any further testing can be carried out.

MICROSCOPY [44-46]

- **Staining method**

Fixation of plant organ-The required samples of different organs were cut and removed from the plant and fixed in FAA (Formalin-5ml + Acetic acid-5ml + 70%Ethyl alcohol-90ml).

Dehydration of specimen-After 24hrs of fixing, the specimen was dehydrated with graded series of tertiary- butyl alcohol.

Infiltration of the specimen-It was carried out by gradual addition of paraffin wax (melting point 58-60 C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

- **Sectioning**

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10-12 μm. Dewaxing of the sections was done by customary procedure. The sections were stained with toluidine blue as per the method published. Since toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to the suberin, violet to the mucilage, blue to the protein bodies etc. Wherever necessary, sections were also stained with safranin, fast-green and iodine (for starch).

- **Paradermal sections**

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of the leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration

employing Jeffrey's maceration fluid was prepared. Glycerine mounted temporary preparations were made for cleared materials. Powdered materials were cleared with sodium hydroxide and mounted in glycerine medium after staining. Different cell components were studied and measured.

- **Photomicrographs**

Microscopic descriptions of tissues were supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright field was used. For the study of crystals, starch grains, lignified cells, polarized light was employed. Since these structures have birefringent property under polarized light they appear bright against dark background.

Magnification of the figure is indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard anatomy books.

POWDER MICROSCOPY [47]

The shade dried leaves of *Operculina turpethum* were powdered well and then the powder was passed through sieve no. 60 and used for powder analysis. The organoleptic characters were observed and to identify the different characteristic features using various staining reagents. Powdered materials of leaves were cleared with NaOH and mounted in glycerine medium after staining with 1% phloroglucinol in 90% ethanol, conc. HCl and glycerine and observed through microscope. Lignified cells can be identified by their pink stain and presence of calcium oxalate crystals can be identified by using polarized light microscope.

QUANTITATIVE MICROSCOPY [48, 49]

Measurement of cell contents of the crude drugs helps in the identification, characterization and standardization. Quantitative microscopy was done as per the procedure.

- **Determination of leaf constants**

The important histological features on the epidermal surfaces of leaves are the stomata, trichomes and palisade cells. The measurements like Stomatal number, Stomatal index, Palisade ratio, Veinislet number and Veinlet termination number are considered to be important parameters in the microscopical evaluation.

- **Stomatal number**

Stomata is epidermal opening covered by two kidney shaped guard cells in dicot leaves. These guard cells, in turn are surrounded by epidermal (subsidiary) cells. Stomata perform the function of gaseous exchange and transpiration in the plants. The nature of the stomata, as well as the stomatal index and stomatal number are the important diagnostic characteristics. Stomatal Number is defined as the average number of stomata per sq.mm of epidermis of the leaf. The actual number of stomata per sq.mm may vary from the leaf to leaf due to difference in environment or climatic conditions of growth. But the ratio of the number of stomata to the total number of epidermal cells in a given area of epidermis is fairly constant for any age of the plant under different climatic conditions.

- **Stomatal Index**

Stomatal Index is the percentage in which the number of stomata forms to the total number of epidermal cells, each stomata being counted as one cell. Stomatal index can be calculated by using following equation.

$$\mathbf{S.I} = \frac{\mathbf{S}}{\mathbf{E+S}} \times 1OO$$

where,

S. I = Stomatal Index

S = Number of stomata per unit area

E = Number of epidermal cells in the same unit area.

- **Vein islet and veinlet termination number**

Vein islet number is defined as the number of vein islet per sq.mm of the leaf surface midway between the midrib and margin. It is also defined as the minute area of the photosynthetic tissue encircled by the ultimate division of the vascular strands. For the study of vein-islet, the leaf material was cut into small bits and boiled in 70%alcohol for 10 minutes to remove chlorophyll pigments. Then the bits were immersed in 10% sodium hydroxide and

kept in thermostat at 400°C for 2-3 days followed by clearing with concentrated chloral hydrate solution. The materials were again washed in distilled water, stained with safranin and mounted in glycerine for observation and photography.

Veinlet termination number is defined as the number of veinlet terminations per sq.mm of the leaf surface midway between the midrib and margin. The cleared leaves were mounted in glycerine after staining with safranin solution. The number of vein islet and veinlet termination was calculated.

LINEAR MEASUREMENTS[50]

- **Linear measurements of length and width of Trichomes**

Measurements of length and width of trichomes in powdered crude drug was done. A little quantity of powder was stained with phloroglucinol and concentrated hydrochloric acid. Powder was spread on the glass slide with dilute glycerine and observed under low power. The full length and width of the trichomes were measured by using eye piece micrometer. With the calibration factor, each was multiplied. The values for 25 trichomes was noted and multiplied by calibration factor and the Average value is detetrmined.

- **Dimensions of Starch grains**

A little quantity of powdered sample was mounted in N/50 Iodine and the diameter of the starch grains were mounted using eye piece micrometer. The values were multiplied by the calibration factor. The average values for diameter were calculated.

PHYSICO-CHEMICAL EVALUATION [51-53]

Shade dried powdered material of leaves of *Operculina turpethum* was used for the determination of physico-chemical constants in accordance with reference given.

- **Ash values**

The ash of any organic material is composed of their non-volatile inorganic components. Controlled incineration of crude drugs results in an ash residue consisting of inorganic materials such as metallic salts and silica. Ash values are helpful in determining the quality and purity of a crude drug in powder form.

- **Total ash**

Determination of ash involves an oxidation of the components of the product. A high ash value is indicative of contamination, substitution, adulteration or carelessness in preparing the crude for marketing. Total ash is designed to measure the total amount of material produced after complete incineration of the ground drug at as low temperature (450°C) as possible to remove all the carbons. The total ash usually consists of carbonates, phosphates, silicates and silica which include both physiological ash, which is derived from the plant tissue itself and non- physiological ash, which is the residue of adhering material to the plant surface. eg., sand and soil.

Procedure

About 2-3gm of air dried crude drug was weighed accurately in tared platinum crucible, incinerated at a temperature not exceeding 450°C. The charred mass was cooled and weighed. The mass was exhausted with charred mass with hot water. The residue was collected on ashless filter paper and incinerated at a temperature not exceeding 450°C. The percentage of ash was calculated.

- **Acid insoluble ash**

It is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

Procedure

To the silica crucible containing total ash obtained, 25ml of hydrochloric acid was added, covered with a watch glass and boiled gently for 5minutes on a burner. The watch glass was rinsed with 5ml of hot water and these washings were added to the crucible. The insoluble matter was collected on ash less filter paper by filtration and this filter paper was rinsed repeatedly with hot water until the filtrate is neutral / free from acid. The filter paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to a constant weight in the muffle furnace and allowed to cool in a dessicator for 30 minutes, and then weighed without delay. The content of acid insoluble ash was calculated as percentage.

- **Water soluble ash**

The ash was boiled for 5minutes with 25ml of water. The insoluble matter was then collected in ash less filter paper. It was washed with hot water and ignited at a temperature not exceeding 450°C. The weight of the insoluble matter was subtracted from the total ash and the difference in weight represents the water soluble ash and then the percentage of water soluble ash with reference to the dried substance was calculated.

- **Sulphated ash**

3g of substance was ignited gently at first in a crucible until the substance was thoroughly charred. Then the residue was cooled, moistened with 1ml of sulphuric acid, heated gently until the white fumes were no longer evolved and ignited at 800±250°C, until all the black particles disappeared. The crucible was allowed to cool, a few drops of sulphuric acid was added and heated. Then it was ignited as before, cooled and weighed. The percentage of sulphated ash with reference to the dried substance was then calculated.

- **Extractive values**

This method determines the amount of active constituents in a given amount of medicinal plant material when extracted with solvents. Extractive values are useful for the evaluation of Phytoconstituents especially when constituents of drug cannot be readily estimated by any other means. Further, these values indicate the nature of the active constituents present in a crude drug.

- **Water soluble extractive value**

Procedure

5g of the air dried coarsely powdered drug was macerated with 100ml of chloroform and water (95ml distilled water and 5ml chloroform) in a glass stoppered conical flask for 24 hours, shaking the contents frequently during the first 6 hours and then allowing to stand for 18 hours. The mixture was filtered rapidly taking precautions again loss of solvents. 25ml of the filtrate was evaporated on a water bath in a tared flat bottomed Petri plate / shallow dish and then 2ml of alcohol was added to dry the residue. The contents were shaken and dried again on water bath. The petriplates were dried at 105°C for 1hour in the

hot air oven and cooled in a dessicator for 30 minutes and weighed. The process was repeated till a constant weight was obtained. The percentage of water soluble extractive with reference to the air- dried drug was calculated.

- **Alcohol soluble extractive value**

The alcohol soluble extractive value is also indicative for the same purpose as water soluble extractive value. The solvent strength of alcohol varies from 20-90% v/v. The solvent strength has to be chosen depending on the nature of drugs to be extracted. The extractive values vary depending upon the strength of alcohol used for extraction.

Procedure

5g of the air dried coarsely powdered drug was macerated with 100ml of alcohol in a glass stoppered flask for 24 hours, shaking the contents frequently during the first 16 hours and allowing to stand for 18 hours. The solution was filtered rapidly taking precaution against loss of alcohol. 25ml of the filtrate was evaporated to dryness on a water bath in a tared flat bottomed Petri-plate/ shallow dish. The petri plates was dried at 105°C for 1 hour in a hot air oven, cooled in a dessicator and weighed. The process was repeated until constant weight was obtained. The percentage of alcohol soluble extractive with reference to the air-dried drug was calculated.

- **Determination of non-volatile ether soluble extractive (Fixed Oil Content)**

A suitably weighed quantity of the crushed air-dried drug was transferred to an extraction thimble and extracted with solvent ether or petroleum ether (B.P.40-60°C) in a Soxhlet for 6 hours. The extract was filtered into a tared evaporating dish, evaporated and dried at 105°C to constant weight. The percentage of non- volatile ether soluble extractive value with reference to the air dried drug was calculated.

- **Determination of volatile ether soluble extractive**

2g of dried powder was accurately weighed and extracted with anhydrous ethyl ether in a continuous extractive apparatus for 20 hours. The ether solution was transferred to tared porcelain dish and evaporated spontaneously. Then it was dried over phosphorous pentoxide for 18 hours, and the total ether extract was weighed. The extract was weighed gradually and dried at 105°C to constant weight. The loss in weight represented the volatile oil portion of the extract.

- **Loss on Drying**

Loss on drying is the loss in weight in % w/w resulting from water and volatile matter of any kind that can be driven off under specified condition.

Procedure

Specified quantity of the substance was transferred in a previously ignited and cooled silica crucible and the substance was evenly distributed by gentle sidewise shaking. The crucible with the contents and the lid were weighed accurately. The loaded uncovered crucible and the lid were placed in the drying chamber (105°C). The substance was heated for a specified period of time to a constant weight. The crucible was covered with the lid and allowed to cool in a dessicator at room temperature before weighing. Finally the crucible was weighed to calculate the loss on drying with reference to the air- dried substance.

- **Determination of Foaming Index**

1gm of coarsely powdered drug was weighed and transferred to 500ml conical flask containing 100ml of boiling water. The flask was maintained at moderate boiling at 80-90°C for about 30 minutes. Then it was cooled and filtered into a volumetric flask and sufficient water was added through the filter to make up the volume to 100ml (V1). The decoction was poured into 10 stopper test tubes (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml and up to 10ml. The volume of the liquid in each tube was adjusted with water to 10ml. The tubes were stoppered and shaken in a length wise motion for 15 seconds, two shakes per second. They were allowed to stand for 15 minutes and the height of the foam was measured. The result is assessed as follows: If the height of foam in every tube is less than 1cm, the foaming index is less than 100. If a height of 1cm is measured in any tube, the volume of the plant material decoction in the tube (a) is used to determine the index. If this tube is the first or second tube in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result. If the height of the foam is more than 1cm in every tube, the foaming index is over 1000. In this case, the determination has to be repeated the determination using a new series of dilution of the decoction in order to obtain a result.

The foaming index is calculated using the following formula:

$$\frac{1000}{a}$$

where, a is the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

- **Determination of Swelling Index**

The swelling index is the volume in ml taken up by the swelling of 1gm of plant material under specified conditions.

Procedure:

A specified quantity of the plant material previously reduced to the required fineness and accurately weighed, into a 25ml glass stoppered measuring cylinder. The internal diameter of the cylinder should be about 16mm, the length of the graduated portion should be about 125mm, marked in 0.2ml division from 0 - 25ml in an upwards direction. 25ml of water was added and shake the mixture thoroughly every 10 minutes for 1 hour. It was allowed to stand for 3 hours at room temperature. The volume in ml occupied by the plant material, including any sticky mucilage was measured. The mean value of the individual determination, related to 1gm of plant material was calculated.

QUALITATIVE AND QUANTITATIVE ESTIMATION OF HEAVY METALS AND INORGANIC ELEMENTS [54]

Plant material plays a vital role in metabolism and osmolity. Presence of elements vary with the soil, climatic conditions etc. There are essential and non-essential elements which may be beneficial or harmful to living things. Non-essential elements like lead, arsenic, cyanide, cadmium and mercury bring about toxic effects resulting in intoxication. Hence qualitative and quantitative estimation of inorganic elements in the plant *Operculina turpethum* were carried out.

- **Qualitative analysis of inorganic elements & heavy metals**

To the ash of the drug material, 50% v/v Hcl was added and kept for 1 hour. It was filtered and the filtrate was used for the following tests.

- **Aluminium**

White gelatinous precipitate of aluminium hydroxide is formed on addition of ammonia solution. It is slightly soluble in excess of the reagent. Fresh precipitate readily dissolves in strong acid and base, but after boiling it becomes difficult in soluble.

- **Arsenic**

Arsenous salts in neutral solution react with solution of copper sulphate to form green precipitate (Scheele's green) which on boiling gives a red precipitate of cupric oxide.

- **Borate**

The mixture obtained by the addition of sulphuric acid and alcohol (95%) to a borate when ignited, burns with flame tinged with green.

- **Calcium**

One drop of dil. Ammonium hydroxide and saturated ammonium oxalate solution was added to 10ml of the above filtrate. A white precipitate of calcium oxalate, soluble in hydrochloric acid but insoluble in acetic acid indicates the presence of calcium.

- **Carbonate**

When treated with dilute acid, effervescence with liberation of CO_2 which is colourless and produces white precipitate in calcium hydroxide solution.

- **Chlorides**

On addition of 3-5ml of lead acetate solution to 5-7ml of the filtrate, if a white precipitate soluble in hot water is formed, it shows the presence of chloride.

- **Copper**

An excess of ammonia, added to a solution of cupric salt, produces first bluish precipitate and then deep coloured solution, if copper is present.

- **Iron**

To 5ml of test solution, few drops of 2% potassium ferrocyanide was added. If a dark blue colouration is observed, it shows the presence of iron.

- **Lead**

Strong solution of lead salts, when treated with Hydrochloric acid, yields a white precipitate which is soluble in boiling water and is re-deposited as crystals when the solution is cooled.

- **Magnesium**

White calcium oxalate precipitate was separated by filtering the above solution. The filtrate was heated and cooled. Solution of sodium phosphate in dilute ammonia solution was added. A white crystalline precipitate indicates the presence of magnesium.

- **Mercury**

Solution of mercuric salts, when treated with sodium hydroxide solution, yields a yellow precipitate.

- **Nitrate**

With solution of ferrous sulphate if no brown colour is produced, then sulphuric acid is added (slow from the side of the test tube), a brown colour is formed at the junction of two liquids, it indicates the presence of nitrates.

- **Phosphate**

5ml of test solution was prepared in nitric acid and few drops of ammonium molybdate were added. It was heated for about 10 minutes and left to be cooled. A yellow crystalline precipitate of ammonium molybdate indicates the presence of phosphate.

- **Potassium**

Few drops of sodium cobalt nitrite solution was added to 2-3ml of the test solution. Yellow precipitate of potassium cobalt nitrite indicates presence of potassium.

- **Silver**

Solution of silver salts, when treated with potassium iodide solution yield a cream coloured precipitate which is insoluble in dilute ammonia solution and in nitric acid.

- **Sulphates**

To 5ml of test solution, lead acetate reagent was added. A white precipitate soluble in sodium hydroxide shows the presence of sulphate.

- **Quantitative estimation of heavy metals and inorganic elements** [55]

The heavy metals and inorganic elements are quantitatively determined by Inductively Coupled Plasma optical emission spectrometry technique the lead (Pb), Arsenic (As), Mercury (Hg), Aluminium (Al), Copper (Co), Calcium (Ca), Iron (Fe), Manganese (Mn), Potassium (K), Sodium (Na), Magnesium (Mg), Zinc (Zn) and Nickel (Ni) by Inductively Coupled Plasma Optical Emission Spectrometry.

Instrumentation parameters:

Instrument model : PE Optima 5300 DV ICP-OES;
Optical System Dual View- axial or radial

Detector system : Charge Coupled Detector, (UV-VISIBLE detector which is Maintaining at -40°C) to detect the intensity of the emission line wavelength range from 165 to 782 nm.

Torch (light source) : Positioned horizontally in the sample compartment along the central axis of the spectrometer optics. Changing from axial to radial viewing in a simple software command and is accomplished by computer control of a mirror located in the optical path. The torch assembly of this system comprises of two concentric quartz tubes.

Standard alumina : 2.0mm in diameter.

Injector

Spray chamber : Scott type.

Nebulizer : Cross flow Gem tip.

- **Preparation of samples by acid digestion method**

Accurately weighed *Operculina turpethum* powder (approximately 50g) was treated with acid mixture of sulphuric acid: water in the ratio of 4:1 in a Kjeldahl flask and heated continuously till the solution is colourless. The sample mixture was then transferred in a 25ml volumetric flask and made up to volume with distilled water.

Blank solution was prepared as above without sample.

The standards of heavy metals and inorganic elements were prepared as per the protocol and the calibration curve was developed for each of them.

II. PHYTOCHEMICAL STUDIES

Phytochemicals are non-nutritive plant chemicals that contain protective and disease preventing compounds. They are involved in many process including ones that help to prevent cell damage, prevent cancer cell replication and decrease cholesterol levels. Some phytochemicals work as anti-oxidants, while others are enzyme inhibitors.

The present Phytochemical investigations of *Operculina turpethum* involves the Qualitative and Quantitative phytochemical evaluation, Fluorescence analysis, Thin Layer Chromatography, Isolation of active constituents by Column chromatography and structural elucidation by spectral studies.

PREPARATION OF EXTRCTS [56]

- **Continuous hot percolation method**

The shade dried, coarsely powdered leaf material of *Operculina turpethum* was extracted successively with Petroleum Ether, Ethyl Acetate and Ethanol using Soxhlet apparatus by continuous hot percolation method for 48 hours. The solvents were completely recovered from the collected extract under reduced pressure by distillation. The concentrated extract were dried on a water bath and preserved in vaccum dessicator for further studies.

- **Cold maceration**

The shade dried, coarsely powdered leaf material of *Operculina turpethum* were charged in an aspirator bottle and extracted with water by cold maceration method for 7days. After decanting and filtering, nearly 80% of the solvent was removed by distillation over boiling water bath and the remaining under reduced pressure. The extract obtained was further dried in vaccum dessicator and the extract was used for further studies.

PRELIMINARY PHYTOCHEMICAL SCREENING [56-58]

Qualitative Phytochemical evaluations were carried out on the different extracts and dried powder to identify the presence of various phytochemical constituents.

- **Detection of Alkaloids**
  - **Mayer's Test**

    The extracts were treated with few drops of Mayer's reagent. Formation of creamy white precipitate indicates the presence of alkaloids.
  - **Dragendroff's Test**

    Extracts were treated with few drops of Dragendroff's reagent. Formation of orange red precipitate indicates the presence of alkaloids.
  - **Wagner's Test**

    Extracts were treated with Wagner's reagent. Formation of brown/ reddish brown precipitate indicates the presence of alkaloids.
  - **Hager's Test**

    Extracts were treated with Hager's reagent. Formation of yellow coloured precipitate indicates the presence of alkaloids.

- **Detection of Carbohydrates**
  - **Molisch's test**

    Filtrate was treated with 2drops of Molisch's reagent in a test tube and 2ml of Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of a violet ring at the junction indicates the presence of carbohydrates.
  - **Benedict's test**

    Filtrate was treated with Benedict's reagent and heated on a water bath. Formation of orange red precipitate indicates the presence of reducing sugars.
  - **Fehling's test**

    Filtrate was hydrolysed with dil. Hydrochloric acid and heated with Fehling's A& B solutions. Formation of red precipitate indicates the presence of reducing sugars.

- **Detection of glycosides**

  Extracts were hydrolysed with dil. Hydrochloric acid and then subjected to test for glycosides.

- **Liebermann-Burchard's Test**

The hydrolysed extracts were treated with chloroform and a few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of brown ring at the junction indicates the presence of glycosides and phytosterols.

- **Modified Borntrager's Test**

The hydrolysed extracts were treated with ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and shaken with equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose pink colour in the ammonical layer indicates the presence of anthranol glycosides.

- **Legal's Test**

Extracts were treated with sodium nitroprusside in pyridine and methanolic alkali. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

- **Detection of saponins**
  - **Foam Test**

Small amount of extract was shaken with little quantity of water. If foam produced persists for 10 minutes, it indicates the presence of saponins.

- **Detection of Phytosterols**
  - **Salkowski's Test**

Extracts were treated with chloroform and treated with a few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

  - **Liebermann-Burchard's Test**

Extracts were treated with chloroform and a few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of brown ring at the junction indicates the presence of glycosides and phytosterols.

- **Detection of fixed oils and fats**
  - **Stain Test**

    Small quantities of extracts were pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oils and fats.

- **Detection of resins**
  - **Acetone-water Test**

    Extracts were treated with acetone. Small amount of water was added and shaken. Appearance of turbidity indicates the presence of resins.

- **Detection of Phenolic acids and Tannins**
  - **Ferric chloride Test**

    Extracts were treated with a few drops of ferric chloride solution. Formation of blackish violet or pinkish red colour indicates the presence of tannins or phenolic compounds.

    Note: Formation of emerald green to brownish black colour could also indicates the presence of phenolic acids or tannins.
  - **Gelatin Test**

    Extracts were treated with few drops of 1% Gelatin solution containing 10% sodium chloride. Formation of white precipitate indicates the presence of tannins.

- **Detection of Proteins and Amino acids**
  - **Xanthoproteic Test**

    The extracts were treated with a few drops of Conc. Nitric acid solution. Formation of yellow colour indicates the presence of proteins.
  - **Ninhydrin Test**

    To the extract 0.25% of Ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.
  - **Biuret Test**

The extracts were treated with 1ml of 10% Sodium hydroxide solution and heated. To this a drop of 0.7% of copper sulphate solution was added. Formation of purplish violet colour indicates the presence of proteins.

- **Detection of Flavonoids**
  - **Alkaline Reagent Test**

    Extracts were treated with few drops of Sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.
  - **Lead acetate Test**

    Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.
  - **Shinoda Test**

    To the alcoholic solution of extracts, a few fragments of magnesium ribbon and Conc. Hydrochloric acid were added. Appearance of magenta colour after few minutes indicates the presence of flavonoids.

- **Test for Terpenoids:**
  - **Noller's Test**

    The extracts were warmed with tin and thionly chloride. Pink colouration indicates the presence of terpenoids.
- **Detection of Gums and Mucilage**

  Extracts were treated with alcohol. Formation of precipitate indicates the presence of gums and mucilage.

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

- **Estimation of Glycosides** [59]

About 10 g of plant material to be analysed is boiled under reflux with 95% ethanol for 30min to destroy enzymes and to extract the glycosides. After chopping the residue of plant material, the extraction is repeated. The combined alcoholic extract is then evaporated to dryness under reduced pressure at a temperature less than 50°C and dry residue is dissolved in water. The solution is precipitated with basic lead acetate and the excess lead

is removed by passing H_2S. Extract is treated with charcoal. The filtrate was concentrated and dried and the weight was determined.

- **Estimation of Flavonoids** [60]

Total flavonoid content was determined by the aluminium chloride colorimetric method, using quercetin as a standard. Aliquots of extracts solutions (1µg/ml) were taken and 0.1ml of $AlCl_3$ (10%), 0.1ml Na-K-tartarate and 2.8ml distilled water were added sequentially. The solution mixture was vigorously shaken. Absorbance at 415nm was recorded after 30 min of incubation. A standard calibration curve was generated at 415nm using known concentrations of quercetin (20, 40, 60, 80,100 µg/ml). The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent / g of sample.

- **Total Phenolic Content** [61]

Standard Gallic acid (10mg) was dissolved in 100ml distilled water in a volumetric flask (100µg/ml of stock solution). From the above stock solution 0.5 to 2.5ml of aliquots were pipette out into 25ml volumetric flask. Then 10ml of distilled water and 1.5ml of Folin-Ciocalteu reagent, diluted according to the label specification to each of the above volumetric flask were added. After 5min, 4ml of 1M Sodium carbonate was added and volume was made up to 25ml with distilled water. At the same time, the plant extract (0.5ml of 1:10mg/ml) in methanol were separately mixed with above reagents. After 30minutes, absorbance at 765nm was recorded and calibration curve for standard was plotted as absorbance Vs concentration. From this graph the amount of Phenolic content was determined.

FLUORESCENT ANALYSIS [62, 63]

Fluorescence analysis was carried out according to the methods in day light and in UV light. The plant powders and extracts were treated with different solvents and the fluorescence was observed in day light and in near and far UV light and results were tabulated.

III. SELECTION OF ACTIVE EXTRACT BY *IN-VITRO* STUDIES

Herbal sources are rich in phytoconstituents many of which are known to have antioxidant properties. Agents with antioxidant property also possess hypolipidemic activity. Pancreatic lipase is an enzyme which plays a major role in promoting absorption of lipids from the intestine. Drugs which inhibit this enzyme may have the potential to act as hypolipidemic agents.

Hence all the extracts of leaves of *Operculina turpethum* were subjected to antioxidant and *in vitro* antioxidant and pancreatic lipase inhibition studies to help in the selection of the best extract which would be taken up for further studies.

ANTIOXIDANT ACTIVITY

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidising agent and thus produces free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves.

- **Effect of Free radicals**

  Some conditions caused by free radicals include

  - Deterioration of the eye lens, which contributes to blindness
  - Inflammation of the joints (arthritis)
  - Damage to nerve cells in the brain, which contributes to conditions such as Parkinson's or Alzheimer's disease
  - Acceleration of the ageing process
  - Increased risk of Coronary heart disease, since free radicals encourage LDL cholesterol to stick to artery walls
  - Certain cancers, triggered by damaged cell DNA.

- **Disease fighting antioxidants**

A diet high in antioxidants may reduce the risk of many diseases including heart diseases and certain cancers. Antioxidants scavenge free radicals from the body cells and prevent the damage caused by oxidation.

1. Hydrogen peroxide radical scavenging (H_2O_2) assay [64]

The ability of the extract to scavenge hydrogen peroxide was determined according to the method of Ruch, Cheng and Klaunig. A solution of hydrogen peroxide (2 mmol/l) was prepared in phosphate buffer (Ph 7.5). Extracts (50-250 µg/ml) were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230nm was determined after 10min against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for background subtraction. The percentage scavenging activity of hydrogen peroxide was calculated using the following formula,

$$\%\text{scavenging activity} = \frac{\text{Abs (control)} - \text{Abs (standard)}}{\text{Abs (control)}} \times 100$$

where Abs (control) : Absorbance of control

Abs (standard): Absorbance of extract/standard

2. Reducing power ability [65]

The reducing power was determined according to the method of Oyaizu.
50-250 µg/ml of Hexane, Ethyl acetate, Ethanol, Aqueous extracts of *Operculina turpethum* in 1ml fractions were mixed with 1ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 1ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. 1ml of 10% Trichloro acetic acid (w/v) was added, the mixture was centrifuged at 2000rpm for 10min. The upper layer solution (2.5ml) was mixed with 2.5ml of deionised water and 0.5ml of fresh Ferric chloride (0.1%). The absorbance was measured at 700nm; a higher absorbance indicates a higher reducing power.

IN VITRO PANCREATIC LIPASE INHIBITORY ACTIVITY [66, 67]

Pancreatic lipase is also known as Pancreatic triacylglycerol lipase is secreted from the pancreas and it is the primary enzyme that hydrolyzes dietary fat molecule in the digestive system converting triacylglyceride substrate into monoglyceride and free fatty acids. Bile salts are secreted from liver and stored in gall bladder where released into duodenum, they coat and emulsify the large fat droplets into small droplets, which allows lipase to break the fat more effectively.

Pancreatic lipase plays an important role in hyperlipidemia by breaking down of lipids for digestion. Agents which inhibit this enzyme are known to have hypolipidemic activity.

- **Extraction of Lipase from Chicken (*Gallus domesticus*) Pancreas**

Pancreas of freshly slaughtered chicken were collected, washed and placed in ice cold sucrose solution (0.01M). The tissue was homogenized in 0.01M sucrose and centrifuged. The supernatant was separated and subjected to ammonium sulphate precipitation (50% saturation). The pellet obtained after centrifugation was dissolved in sucrose solution and again saturated to 50% ammonium sulphate saturation and centrifuged. The pellet obtained was dissolved in phosphate buffer and used as enzyme.

- **Determination of Chicken Pancreatic Lipase Activity**

The chicken pancreatic lipase activity was determined by incubating an emulsion containing 8ml of olive oil, 0.4ml of phosphate buffer and 1ml of chicken pancreatic lipase for an hour in rotary shaker, the reaction was stopped by addition of 1.5 ml of a mixture solution containing acetone and 95% ethanol (1:1). Appearance of pink colour from yellow colour shows the liberated fatty acids, which was determined by titrating the solution against 0.02M NaOH (standardized by 0.01M oxalic acid) using phenolphthalein as an indicator.

- **Lipase inhibitory activity of various extracts of *Operculina turpethum***

Lipase inhibitory activity of different concentrations of extracts was tested by mixing 25-100µg/ml of each concentration of extract, 8ml of olive emulsion and 1ml of chicken pancreatic lipase followed by incubation for 60 minutes. The reaction was stopped by adding 1.5ml of a mixture containing acetone and 95% of ethanol (1:1). The liberated fatty acids were determined by titrating the solution against 0.02M NaOH (standardized by 0.01M oxalic acid) using phenolphthalein as indicator.

Percentage inhibition of lipase activity was calculated using the formula

$$\textbf{Lipase inhibition} = \frac{A-B}{A} \times 100$$

where A is titre value without extract

B is activity of lipase when incubated with the extract.

The extract which has the highest inhibitory potential was selected and subjected for TLC, *in vivo* hypolipidemic evaluation by diet induced hyperlipidemia model and isolation, characterization of active component.

1V. CHROMATOGRAPHIC PROFILE OF SELECTED ETHANOLIC EXTRACT[68,69]

Chromatography is a separation technique in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase.

- **Principle**

The principle of separation is adsorption. One or more compounds are spotted on a thin layer of adsorbent coated on a chromatographic plate. The mobile phase (developing solvent) flows through because of capillary action (against gravitational force). The compounds move according to their affinities towards the adsorbent. The component with more affinity towards the stationary phase travels slower. The component with lesser affinity towards the stationary phase travels faster and eluted first.

- **TLC plates**

  The precoated silica gel plates were used.

- **Selection of mobile phase**

  The specific solvent system suggested for each phytoconstituent was used.

- **Sample application**

The suitably diluted extract was applied to a TLC plate as a spot of as small a diameter as possible. The sample volume employed with normal TLC plates was 1 to 2 ml. The sample was applied with a capillary tube, discharging the contents of the pipette by surface tension, touching the surface of the plate. The plate was kept in a TLC chamber containing saturated solvent system. After the movement of the solvent along with the sample against gravitational force due to capillary action to a suitable distance, the plate was removed and air-dried. The Rf value was calculated using suitable detecting agent.

- **Detecting agent**

  UV 254 nm and 365 nm.

V. ISOLATION AND CHARACTERIZATION OF PHYTOCONSTITUENTS BY COLUMN CHROMATOGRAPHY [70-72]

Column chromatography is suitable for the physical separation of gram quantities of material. Solvent act as the mobile phase while a finely divided solid surface act as the stationary phase. The stationary phase will adsorb the componenets of the mixture to varying degrees. As the solution containing the mixtures passes over the adsorbent, the components are distributed between the solvent and adsorbent surface. This process may be described by three-way equilibrium between the sample, the solvent and the adsorbent.

Selection of solvents requires a balancing solvent and compound polarity. For most separations, the solvent should be less polar than the compounds. The compounds must

also be soluble in the solvent so they are not permanently adsorbed. The elutropic series (order of polarity) for silica gel and alumina is as follows: Hexane / Petroleum ether < Carbon tetrachloride < toluene < dichloromethane < chloroform < diethyl ether < ethyl acetate < acetone < propanol < ethanol < methanol < acetic acid < water.

MATERIALS AND METHODS

Extract used : Ethanolic extract

Method : Wet packing method

Packing material : Silica gel G 70 – 325

Procedure

The ethanolic extract was subjected to silica gel column chromatography for the isolation of phytoconstituents.

An appropriate column sized 2.5cm diameter and 60cm length was used. It was washed with water and rinsed with acetone and then dried completely. A small quantity of pure cotton was placed at the bottom of column. The weighed quantity of silica gel was made into a slurry with hexane and poured in to the column with stirring until it was uniformly wet and the column was allowed to settle down. Meanwhile about 10gm of extract was weighed and mixed with silica gel until it became free flowing. It was packed in the column and a piece of cotton was placed above it. The column was then run with solvents in increasing polarity such Hexane, Hexane: Chloroform, Chloroform, Chloroform : Ethyl acetate, Ethyl acetate, Ethyl acetate: Ethanol and Ethanol. Fractions were collected and analysed by using TLC plate with suitable mobile phase.

THIN LAYER CHROMATOGRAPHY

The fractions collected were subjected it to TLC, the fractions with same Rf values were combined and the solvent was evaporated. The solvent system used for TLC was Ethyl actate: Methanol: Water (81: 11: 8). The development chromatogram was observed under UV fluorescence and also derivatised with detecting agents.

VI. SPECTRAL STUDIES

The structure of the single isolated compound was derived by the following spectral analysis.

- Infra-red spectroscopy
- Nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C NMR)
- Mass spectroscopy

Spectroscopy is the measurement and interpretation of electromagnetic radiation absorbed or emitted when the molecules or atoms or ions of a sample move from one energy state to another energy state. It measures the changes in rotational, vibrational & electronic changes.

UV SPECTROSCOPY

Ultraviolet absorption spectra is the transition of electron within a molecule or from a lower to a higher electronic energy level. From the position of absorption peak the nature of electrons, saturation and unsaturation of molecule and presence of hetero atoms like S, N, O or halogens are determined.

INFRA-RED SPECTROSCOPY

It is the absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam. It is carried out for the determination of chemical functional groups in the sample.The commonly used sampling techniques for solid sample include pressed pellet technique and for liquid sample Nujol mull technique. The most useful I.R region lies between 4000 – 670 cm^{-1}. Vibrations (stretching and bending) that cause change in dipole moment and bond length when IR radiation of suitable frequency is applied, then the vibrations are infra-red active.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Principle of NMR is based upon the spin of the nuclei in an external magnetic field. In the absence of magnetic field, the nuclear spins are oriented randomly. Once a strong magnetic field is applied, they reorient their spins i.e. aligned with the field or against the

field. Orientation parallel to alignment of applied force is lower in energy. When nuclei are irradiated with RF radiation, the lower energy nuclei flip to high state and nuclei said to be in resonance, hence the term nuclear magnetic resonance.

- **Hydrogen-1 NMR**

Proton NMR (also Hydrogen-1 or ^{1}H NMR) is the application of nuclear magnetic resonance in NMR spectroscopy with respected to hydrogen-1 nuclei within the molecule of a substance, in order to determine the structure of its molecules. Proton NMR spectra of most organic compounds are characterized by chemical shifts in the range +14 to -4 ppm and by spin- spin coupling between protons.

- **Carbon-13 NMR**

^{13}C NMR is an important tool in chemical structure elucidation in organic chemistry. ^{13}C NMR detects only the ^{13}C isotope of carbon, whose natural abundance is only 1.1%, because the main carbon isotope, ^{12}C, is not detectable by NMR since it has zero net spin. In contrast to ^{1}H NMR, the intensities of the signals are not normally proportional to the number of equivalent ^{13}C atoms and are instead strongly dependent on the number of surrounding spins (typically ^{1}H). Spectra can be made more quantitative, if necessary, by allowing sufficient time for the nuclei to relax between repeat scans.

MASS SPECTROSCOPY

Mass spectra is a positive ion spectra which is concerned with the electron ionization, subsequent fragmentation of molecules, determination of mass to charge ratio (m/e) and relative abundances of ions which are produced.

Mass spectroscopy is used to determine the molecular weight of the compound. In an electron impact mass spectrometer, a high energy beam of electrons is used to displace an electron from the organic molecule to form a radical cation known as the molecular ion. If the molecular ion is too unstable then it can fragment to give other smaller ions. The collection of ions is then focussed into a beam accelerated into the magnetic field and deflected along circular paths according to the masses of the ions. By adjusting the magnetic field, the ions can be focussed on the detector and recorded.

VII. PHARMACOLOGICAL STUDIES

IN VIVO HYPOLIPIDEMIC ACITVITY [73]

- **Experimental animals**

Healthy male Wistar Albino rats (200-250g) were procured from Animal Experimental Laboratory, Madras Medical College, Chennai-03. Animal House. The study was approved by the Institutional Animal Ethical Committee which is certified by the committee for the purpose of control and supervision of experiments of animals, India (CPCSEA).

- **Approval number: 7/243 CPCSEA dated 22.11.2013**

The animals were kept individually in clean and dry metallic cages and maintained in a well ventilated animal house at a temperature of 22 ± 2°C and for 12 h light and 12 h dark cycle. The animals fed with standard pellet diet and water *ad libitum.*

Prior to evaluation of the hypolipidemic activity, the extract was subjected to Acute toxic studies in order to establish the safety of the extract.

1. **Acute toxicity studies**

The Organisation of economic co-operation and development (OECD) guidelines 423 was followed the acute toxic class method is a stepwise procedure which was 3 animals of single sex per step. Depending on the mortality and morbidity status of the animals, on average of 2 to 4 steps may be necessary to allow judgement on the acute toxicity of the test substance. This procedure results in the use of minimal number of animals while allowing for acceptable data based scientific conclusion.

Literature review shows that the plant *Operculina turpethum* is not toxic till the dose level of 2000 mg/kg [74]. Hence a starting dose level of 2000 mg/kg of ethanolic extract of leaves of *Operculina turpethum* was used. After oral administration, animals were observed at an hourly basis for the first 4 h and periodically for 24 h to assess the general behaviour and were further observed for 72 hrs for toxic symptoms and mortality of the animal.

The flow chart in figure depicts the procedure adopted for this method.

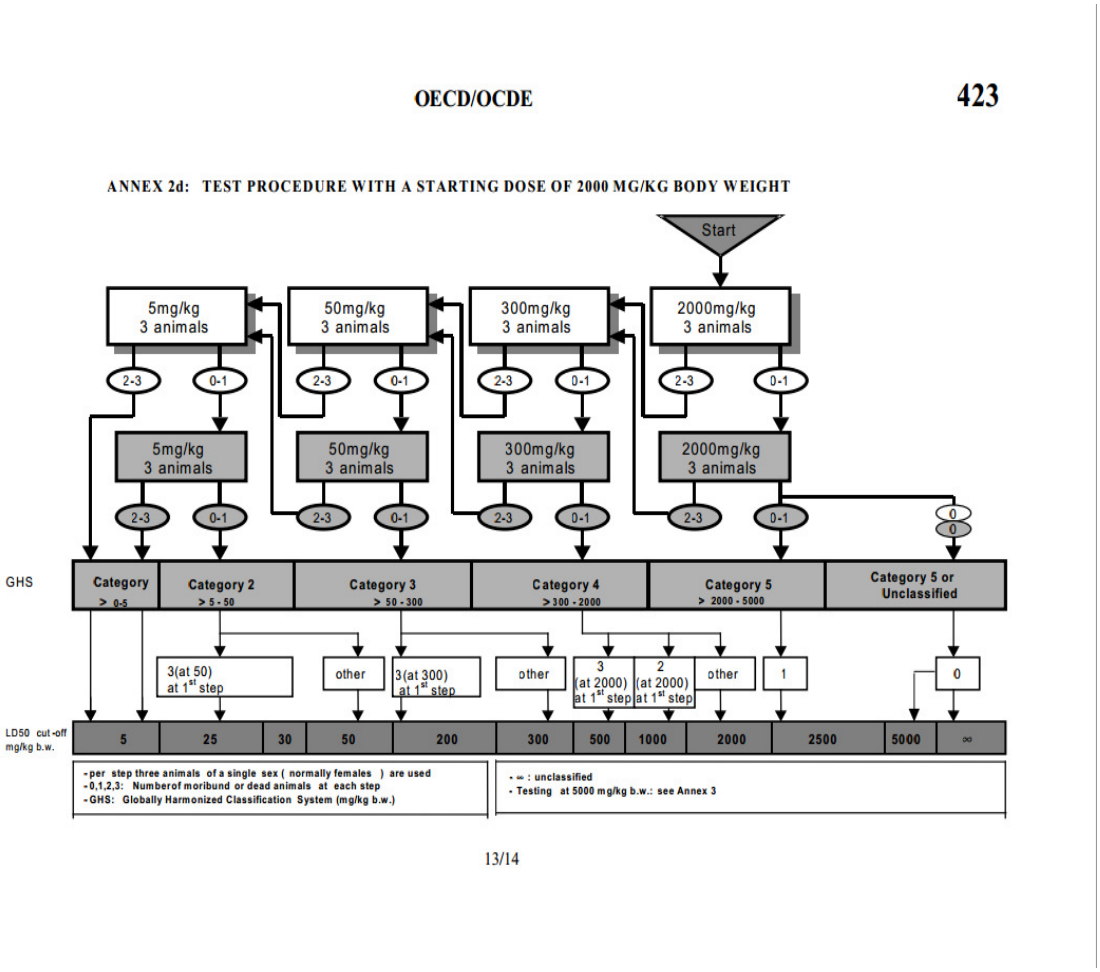


Fig 2: Flow chart for acute toxicity studies

2. Hypolipidemic activity using diet induced hyperlipidemia model

➢ Chemicals

Atorvastatin and sodium taurocholate was obtained as a gift sample from Fourrt's India, Chennai, and Teva Pharmaceuticals, Bangalore. Cholesterol obtained from Microfine lab, Chennai. All the chemicals used in the study were of analytical grade.

- **Procedure**

Hyperlipidemia in rats was induced by administration of high cholesterol diet (2% Cholesterol, 1% Sodium cholate, and 2% Coconut oil) for 30 days in standard rat chow diet. All animals were weighed and divided into five groups, each group containing six animals.

Table 4: Diet Induced Hyperlipidemia Model

Group	Name of the group	Treatment schedule
Group 1	Normal control	Normal food and vehicle.
Group 2	Cholesterol control	Cholesterol 400 mg/kg p.o for 30 days.
Group 3	Standard control	Cholesterol 400 mg/kg p.o for 30 days and Atorvastatin 1 mg/kg p.p from day 16 to 30.
Group 4	Test group 1	Cholesterol 400 mg/kg p.o for 30 days and ethanol extract of *Operculina turpethum* (200 mg/kg) p.o from day 16 to 30.
Group 5	Test group 2	Cholesterol 400 mg/kg p.o for 30 days and ethanol extract of *Operculina turpethum* (400 mg/kg) p.o from day 16 to 30.

Initial body weight of the animals and the body weight at the end of the study period were recorded. The rats were eventually sacrificed by cervical dislocation after 48 hours of last dose. The blood samples to be collected by Retro orbital puncture with glass capillary and allowed to clot for 30 min at room temperature. The clear serum was separated by centrifugation at 2500 rpm for 10 min and used for the determination of bio-chemical parameters.

- **Parameters evaluated**
  - **Bio-chemical analysis**

The serum was analysed for Total cholesterol, Triglycerides, High density lipoprotein (HDL), Low density lipoprotein (LDL), Very low density lipoprotein (VLDL), Serum glutamate oxaloacetate transferase (SGOT), and Serum glutamate pyruvate transferase (SGPT) by using standard methods.

Atherogenic index (AI) and LDL-C/ HDL-C ratio were also calculated. The AI was calculated using the formula

AI = (Total cholesterol — HDL-C) / HD.

- **Statistical Analysis**

All the data were expressed as mean ± S.E. The significance of the difference between the means of the test groups and control group was established by One way ANOVA followed by *post hoc* Dunnett's t test for variance using IBM SPSS statistics, version 21. A P value of less than 0.01 ($P<0.01$) was considered significant.

- **Histopathological studies**

A small portion of aorta was taken from each group and was immediately put in 10% formasal (formalin diluted to 10% with normal saline) and then it was processed. Sections were stained with Ehrlich's haematoxylin and Eosin to find out the Atherosclerotic lesions microscopically.

8. RESULTS AND DISCUSSION

PHARMACOGNOSTICAL STUDIES

The results of Pharmacognostical studies are as follows

LEAVES

Macroscopical features

Leaf	: Simple, alternate
Shape	: Ovate, oblong, cordate
Size	: 5-15cm long; 1.3-7cm broad
Texture	: Scabrous
Apex	: Acuminate
Colour	: Green, dull green
Taste	: Bitter
Margin	: Sinuate and dentate
Surface	: Both surfaces are smooth
Odour	: Characteristic

Fig. 3: Morphology of the leaves of *Operculina turpethum*

Fig. 4: Exomorphic features of the leaves and flowers *Operculina turpethum*

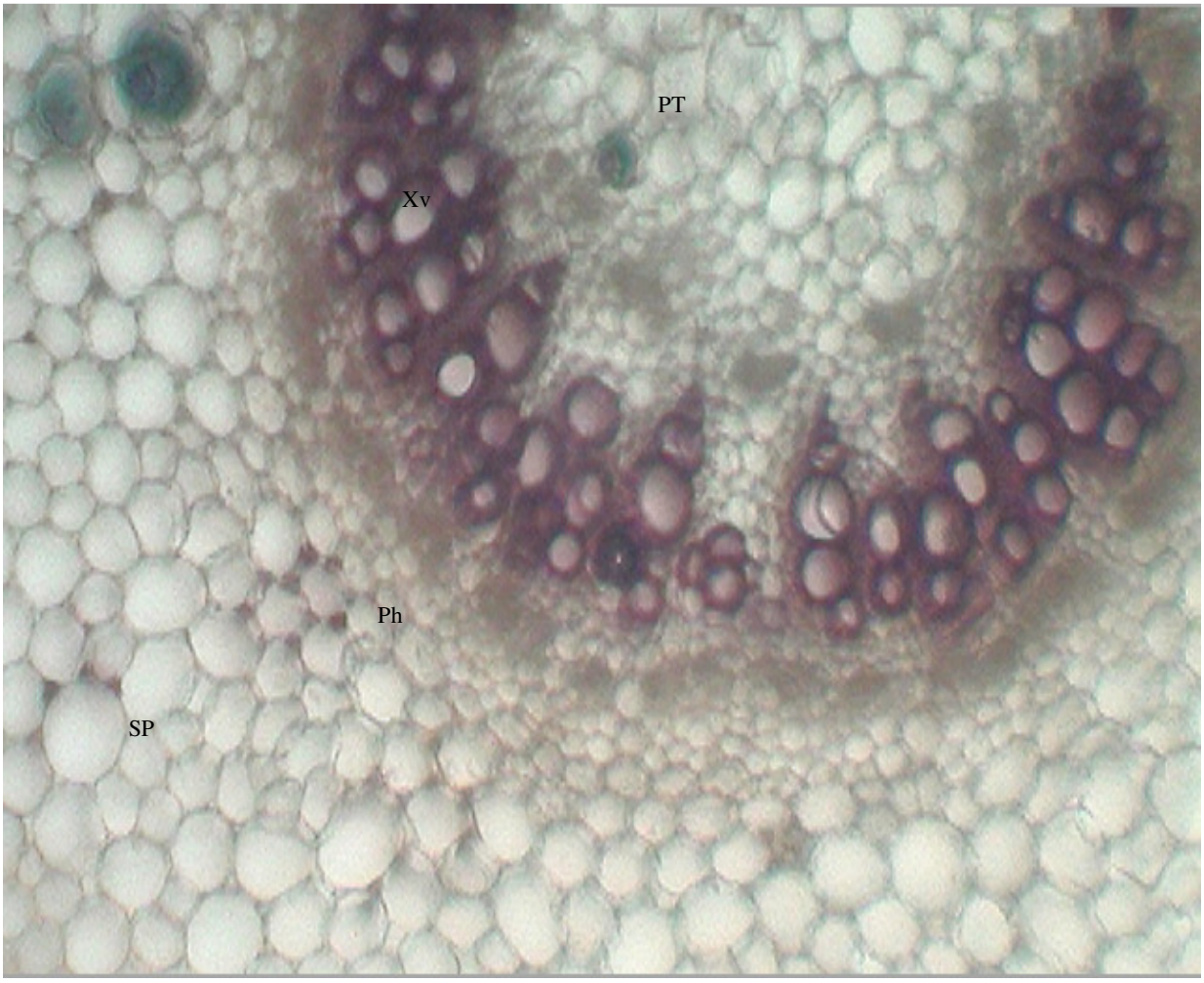
Microscopical features

T.S OF LEAF

Ventral surface of the leaf is dome shaped. Epidermis is composed of single layer of oval shaped thick walled cells. Many covering trichomes are seen in upper and lower side. Epidermis is followed by wide cortical region composed of 8- 12 layers of parenchyma cells on ventral side and 5-10 layers of cells on dorsal side. The parenchyma cells are similar, almost isodiametric to circular in shape. The vascular bundle in the centre is arc shaped and collateral. The phloem surrounds xylem entirely; the xylem cells are lignified and thick walled.

TR- Trichome Xv- Xylem Ph- Phloem SP- Secondary phloem AbE- Abaxial epidermis

Fig. 5: TS of leaf through midrib (10X)



Xv- Xylem PT- Palisade tissue Ph- Phloem SP- Secondary phloem

Fig. 6: Midrib entire view (40X)

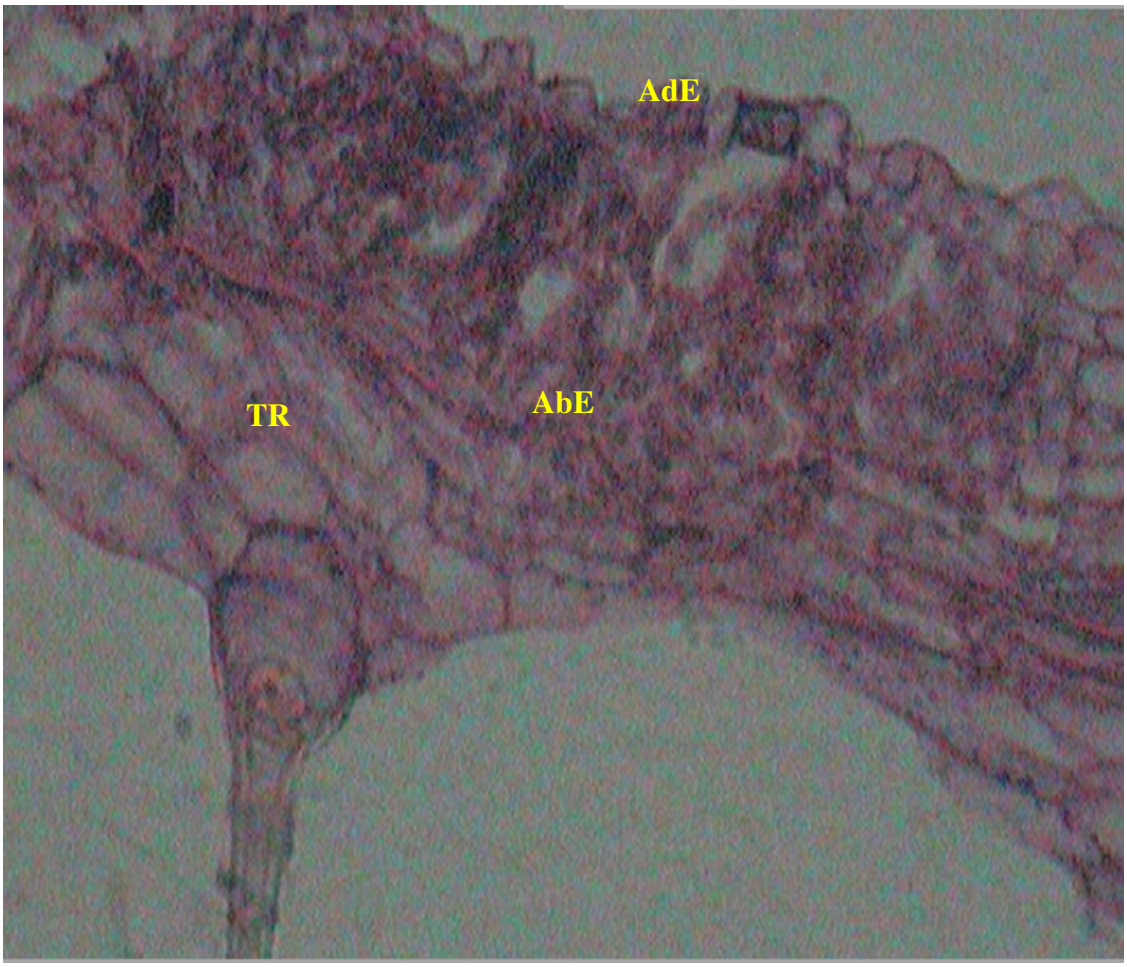
TS OF LAMINA

Lamina is dorsi-ventral in nature, composed of double layered palisade tissues on the upper side and spongy tissues on the lower side. The epidermis is made up of thick walled cells elongate to form covering trichomes with blunt ends. Covering trichomes are 1 to 4 celled present on both the side of the lamina. Few glandular trichomes are seen only in the lower epidermis. Below upper epidermis elongated palisade cells and spongy parenchyma cell layers are seen. Spongy mesophyll consists of circular to elliptic cells with intercellular spaces. Micro rosette type of calcium oxalate crystals are seen in the mesophyll. Anisocytic stomata cells are seen on both the surfaces.

AdE- Adaxial Epidermis AbE- Abaxial epidermis PC- Parenchyma cells

TR- Trichomes

Fig. 7: TS of lamina (10X)

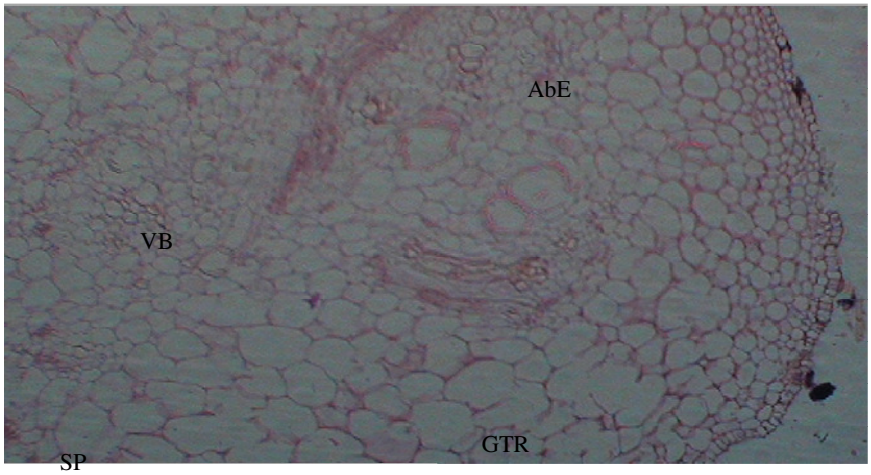


AdE- Adaxial Epidermis, AbE- Abaxial Epidermis, TR- Trichome

Fig. 8: Lamina enlarged (40X)

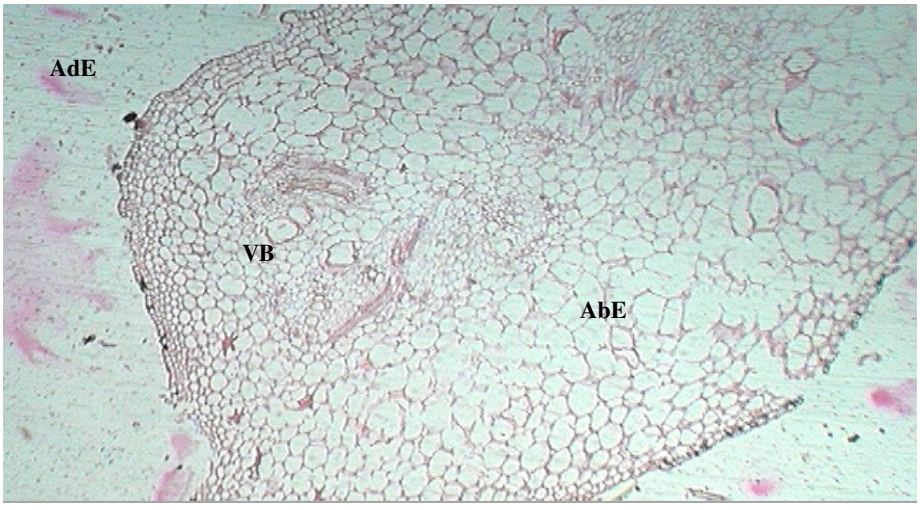
TS OF PETIOLE

Dorsal surface is dome shaped. The epidermis composed of single layered, oval cells with many covering trichomes. Below the epidermis, large cortex is observed with 7-14 layers of parenchyma cells. The cortical cells are polyhedral with intercellular spaces. Vascular bundle present towards the dorsal side. Xylem is surrounded by phloem. Xylem composed of xylem parenchyma, Xylem vessels, tracheids and fibres. Phloem composed of phloem parenchyma, phloem fibre, sieve elements and companion cells. Covering trichomes are present on dorsal and ventral side.



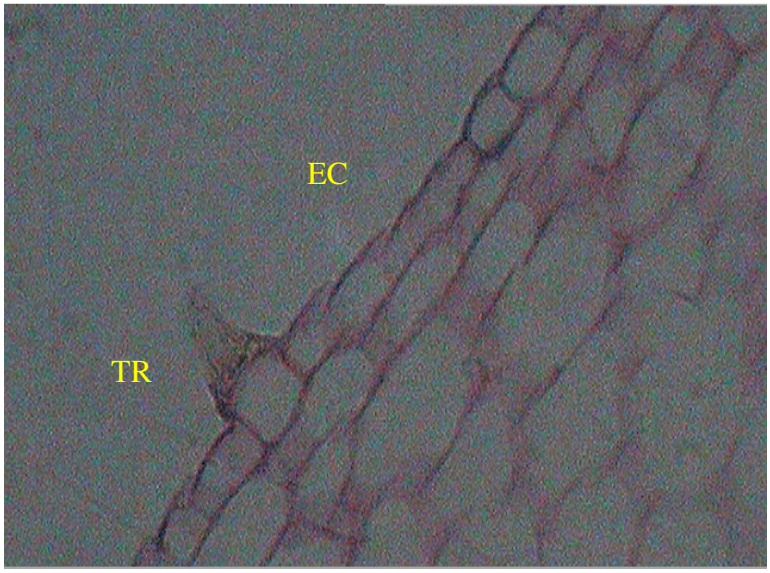
VB- Vascular bundle GTR- Glandular trichomes SP- Secondary phloem AbE- Abaxial epidermis

Fig. 9: TS of petiole (10X)



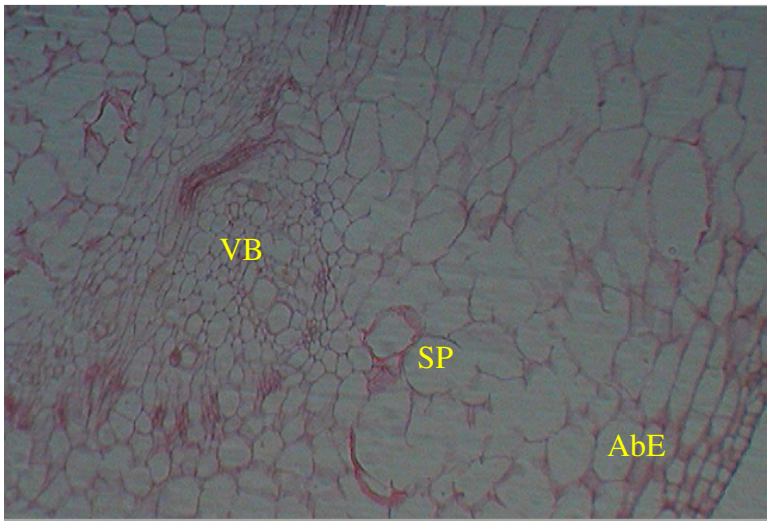
AbE- Abaxial epidermis VB- Vascular bundle AbE- Abaxial epidermis

Fig. 10: TS of petiole (4X)



TR- Trichomes EC- Epidermal cells

Fig. 11: Petiole showing trichome (40X)



VB- Vascular bundle, SP- Secondary Phloem, AbE- Abaxial Epidermis

Fig. 12: Petiole enlarged (40X)

Powder microscopy

The powder microscopy of *Operculina turpethum* showed the following characters

ORGANOLEPTIC CHARACTERS

Nature : Coarse

Colour : Dull green colour

Odour : Characteristic odour

Taste : Characteristic taste

Microscopical characters

The powder of the leaves shows the following inclusions when examined under the microscope which are of diagnostic values.

- Unicellular covering trichomes
- Presence of stomata is very few in the epidermis
- Parenchyma cells with trichomes

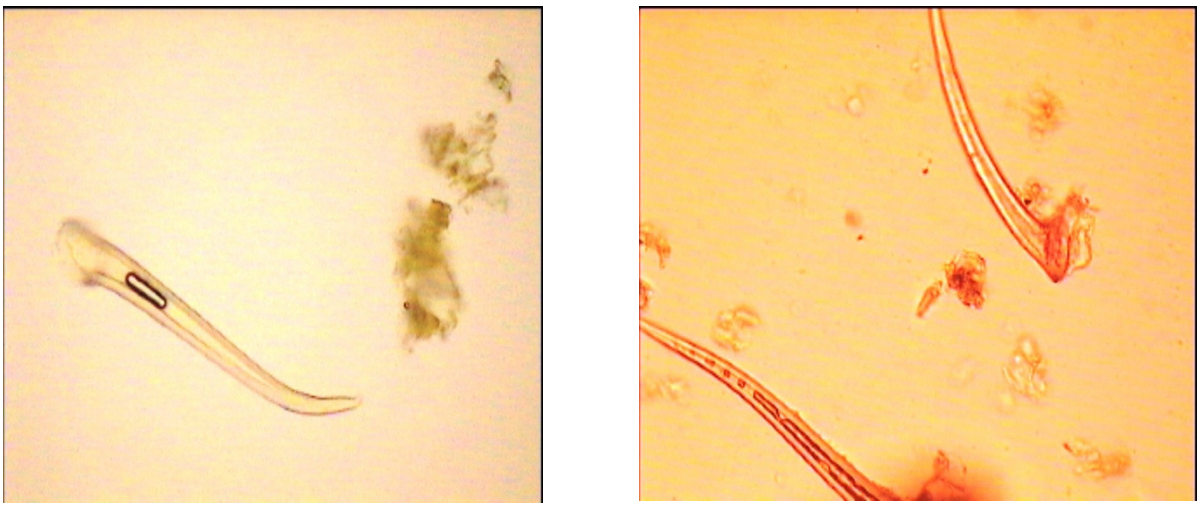


Fig. 13: Shows unicellular elongated Trichomes

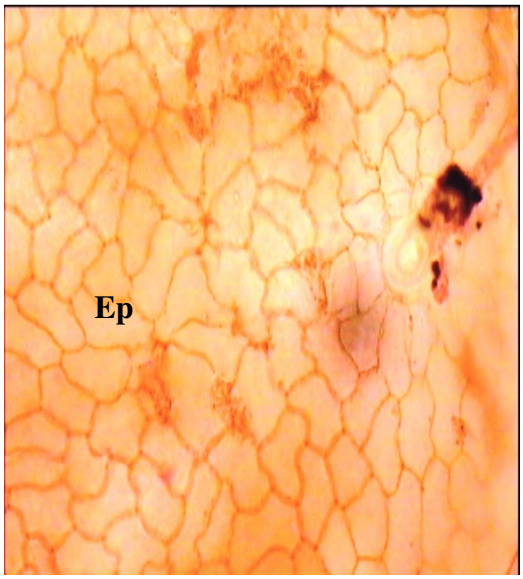


Fig . 14: Ep- Epidermal cells

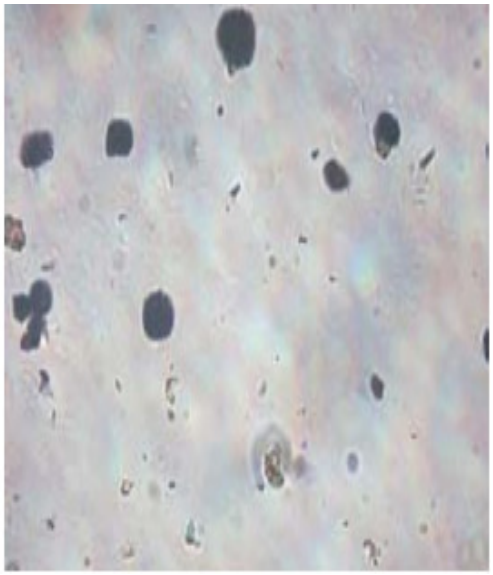


Fig. 15: Sg- Starch grains

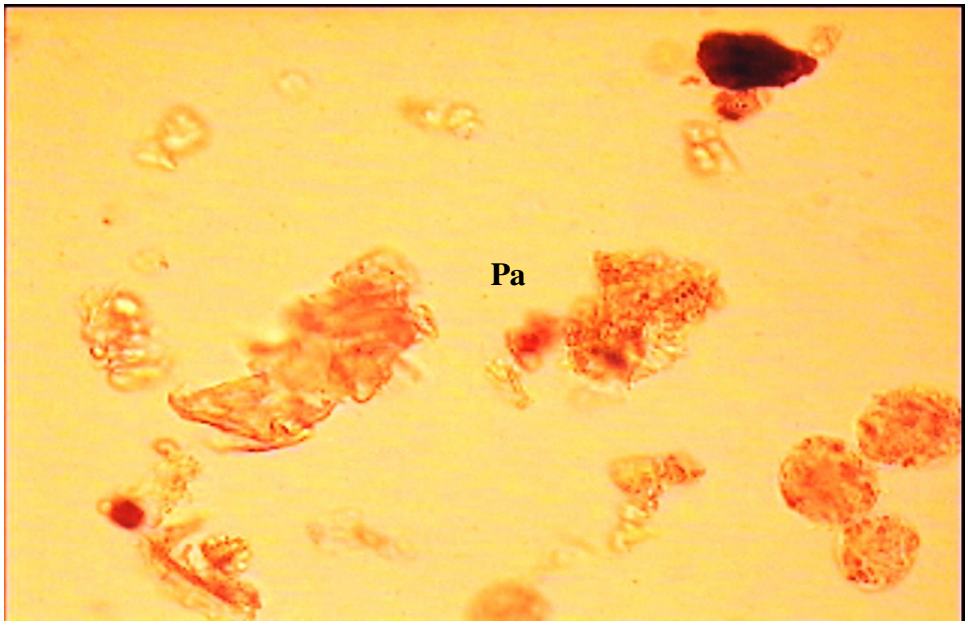
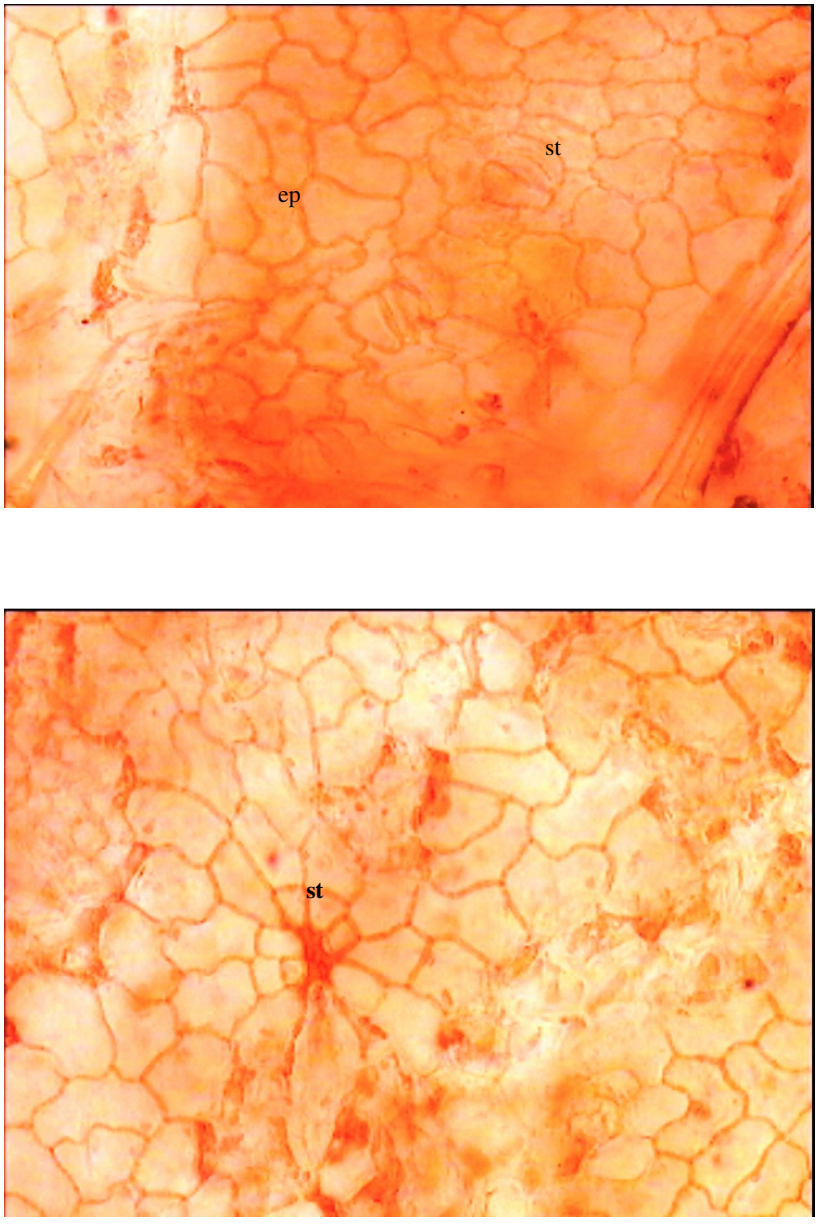


Fig .16: Pa- Parenchyma cells

STOMATA

The stomata are seen on the adaxial and abaxial epidermis. A stomata present in the leaf is Anomocytic.

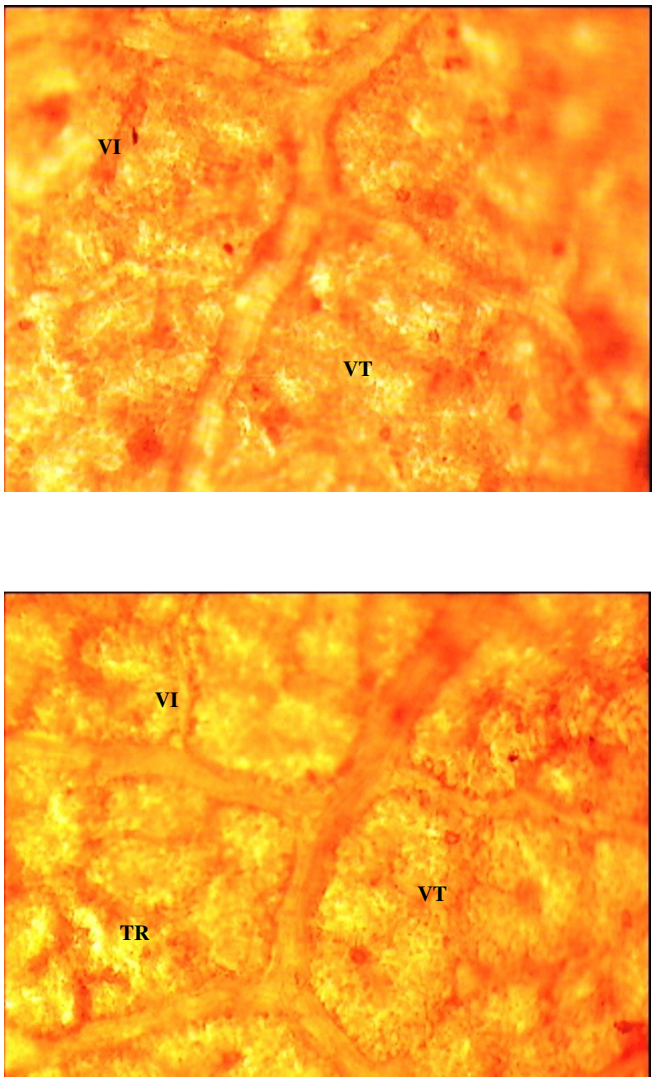


St- stomata ep-epidermis

Fig. 17: Epidermal cells and stomata

VENATION PATTERN

The veins are seen in surface view of cleared lamina, exhibits densely reticulate venation system. The major lateral veins are thick. The veinlets are also fairly thick. The shape and size of the islets are variable. The vein-terminations are thick and well developed. They are either branched or unbranched.



Vi-vein islet vt-veinlet termination tr-trichome

Fig. 18: Venation pattern

Quantitative microscopy

The results of leaf constants like stomatal number, stomatal index, vein islet and veinlet termination number and also the linear measurements of trichomes and starch grains are reported in Tables 5, 6, and 7.

Table 5: Leaf constants

Parameters	Values in sq mm
Stomatal number	75
Stomatal index	160
Vein islet number	65
Veinlet termination number	42

Table 6: Linear measurements of trichomes

Parameters	Minimum (µm)	Average (µm)	Maximum (µm)
Length	217	461	705
Width	12.8	25.6	38.4

Table 7: Linear measurements of starch grains

Parameters	Minimum (µm)	Average (µm)	Maximum (µm)
Diameter	13.3	19.95	33.25

Various physico-chemical parameters were evaluated and the results are given in Table8.

Table 8: Physicochemical constants of the leaves of *operculina turpethum*

S.NO	PARAMETERS	PERCENTAGE (%W/W)
I	**Ash Values**	
1	Total ash	8.34±0.25
2	Acid insoluble ash	2.33±0.12
3	Water soluble ash	4.09±0.2
4	Sulphated ash	11.53±0.03
II	**Extractive Values**	
1	Water soluble extractive	7.06±0.32
2	Alcohol soluble extractive	12.13±0.19
3	Ether soluble(non-volatile) extractive	3.89±0.14
4	Ether soluble(volatile) extractive	3.52±0.08
III	**Loss on drying**	3.12±0.27
IV	**Swelling index**	NIL
V	**Foaming index**	<100

Values are expressed as a mean ± SD (n=3)

Qualitative and quantitative estimation of inorganic elements and heavy metals were analysed in the leaf powder and the results were tabulated in Table 9

Table 9: Qualitative analysis of heavy metals and inorganic elements

S.NO	ELEMENTS	OBSERVATIONS
1	Aluminium	+
2	Arsenic	-
3	Borate	-
4	Calcium	+
5	Carbonate	-
6	Chloride	-
7	Copper	+
8	Iron	+
9	Lead	-
10	Magnesium	+
11	Mercury	-
12	Nitrate	-
13	Phosphate	-
14	Potassium	+
15	Silver	-
16	Sulphate	+

+ ive indicates presence, - ive indicates absence

The powder shows the presence of Aluminium, Copper, Iron, Magnesium, Potassium and Sulphur. The quantitative estimation of these elements was carried out and the results are tabulated in Table 10.

Table 10: Quantitative estimation of inorganic elements

S.NO	INORGANIC ELEMENTS	CONTENT (µg/mg)
1	Aluminium	0.951
2	Copper	0.186
3	Calcium	0.254
4	Iron	1.718
5	Magnesium	0.053
6	Potassium	0.117
7	Sulphate	0.659

The quantitative estimation of heavy metals (As, Cd, Pb) showed that all these were within the specified limits. The results were tabulated in Table 11.

Table 11: Quantitative estimation of heavy metals

S.NO	ELEMENT	RESULTS (ppm/ml)	SPECIFICATION
1	Arsenic	0.002	Not more than 5.0 ppm
2	Cadmium	0.024	Not more than 0.3 ppm
3	Lead	0.070	Not more than 10 ppm

DISCUSSION

The macroscopic and microscopic features of the leaves of *Operculina turpethum* were studied. Some of the characteristic features of the leaves are:

- Powder microscopy helps in identification of small fragments and adulterants.
- The physico-chemical analysis was performed to find out the confirmation and quality of the crude drug.
- Ash value is an important parameter to find the low grade product and exhausted drugs. A high ash value is an indicator of substitution, contamination and adulteration. The total ash value was found to be 8.34%w/w. The acid insoluble, water soluble and sulphated ash was found to be 2.33%, 4.09% and 11.53% respectively.
- Extractive value is helpful for the prediction of nature of constituents. The water soluble, alcohol soluble, ether soluble (non-volatile), ether soluble (volatile) was found to be 7.06%, 12.13%, 3.89% and 3.52% respectively.
- Loss on drying determines the amount of volatile matter present and it was found to be 3.12%. There is no swelling index which indicates that the powder was devoid of sticky mucilage and the foaming index was found to be less than 100.
- Presence of inorganic elements and heavy metals were analysed qualitatively and quantitatively. The inorganic metal analysis showed the absence of toxic metals. The quantification of heavy metals shows the presence of elements within the limit.

From the above results, a complete profile of pharmacognostical parameters was evolved.

PHYTOCHEMICAL STUDIES

The percentage yield of various extracts of leaves of *Operculina turpethum* are shown in Table 12

Table 12: Percentage yield of successive solvent extracts of the leaves of *Operculina turpethum*

S.NO	EXTRACTS	METHOD OF EXTRACTION	PHYSICAL NATURE	COLOUR	YIELD %w/w
1	Hexane	Soxhlet extraction	Sticky	Blackish green	3.18
2	Ethyl acetate		Semisolid	Dark green	8.93
3	Ethanol		Sticky	Greenish black	12.09
4	Aqueous	Maceration	Sticky	Greenish brown	7.12

The ethanolic extract showed the maximum yield which is more when compared to other extracts and its percentage yield was found to be 12.09%w/w.

Qualitative phytochemical analysis for the various extracts of leaves of *Operculina turpethum* was performed and tabulated in Table- 13.

Table 13: Preliminary phytochemical analysis of powder and extracts of leaves of *Operculina turpethum*

S.NO	PHYTOCHEMICAL TESTS	POWDER	HEXANE EXTRACT	ETHYL ACETATE EXTRACT	ETHANOL EXTRACT	AQUEOUS EXTRACT
1	Alkaloids	+	-	-	+	-
2	Carbohydrates	+	-	-	+	+
3	Flavonoids	+	-	+	+	-
4	Glycosides	+	-	+	+	+
5	Fixed oil and fats	-	-	-	-	-
6	Phytosterols	+	+	+	+	-
7	Phenolic compounds	+	-	+	+	+
8	Proteins and amino acids	-	-	-	-	-
9	Resins	-	-	-	-	-
10	Saponins	+	-	+	+	+
11	Terpenoids	+	-	+	+	-
12	Tannins	+	-	+	+	+
13	Gums and mucilage	+	-	-	-	+

+ ive indicates presence, - ive indicates absence

The ethanol extract showed the presence of maximum phytoconstituents such as alkaloids, flavonoids, glycosides, carbohydrates, phytosterols, phenols, saponins, terpenoids and tannins. All these phytoconstituents were also seen to be present in crude powder drug. The ethyl acetate extract also showed the presence of most of these phytoconstituents except alkaloids and sugars. The hexane extract also showed only the presence of phytosterols and the aqueous extract showed absence of alkaloids, flavonoids, phytosterols and terpenoids and showed the presence of other phytoconstituents along with gums and mucilage.

The Fluorescence analysis of the powder and the various extracts are given in Tables 14 and 15.

Table 14: Fluorescence analysis of various extracts of the leaves of *Operculina turpethum*

S.NO	TREATMENT	DAY LIGHT	UV LIGHT	
			SHORT 254nm	LONG 365nm
1	Powder	Green	Green	Brown
2	Powder + water	Pale Green	Pale Green	Green
3	Powder + 1N HCl	Greenish Yellow	Light green	Yellowish green
4	Powder + 1N HNO_3	Pale Brown	Brownish Green	Green
5	Powder+Acetic acid	Green	Pale Green	Light green
6	Powder+1N NaOH	Yellowish Green	Pale brown	Light green
7	Powder+1NAlc NaOH	Pale Green	Green	Brownish Green
8	Powder+ 1N KOH	Dark Green	Yellowish green	Green
9	Powder+1N Alc KOH	Green	Brownish Yellow	Dark Green
10	Powder+H_2SO_4	Brownish Green	Green	Green
11	Powder + Ammonia	Pale Green	Green	Light Greeen
12	Powder + Iodine	Brownish Black	Green	Brown
13	Powder + $FeCl_3$	Blackish brown	Dark Green	Dark Brown
14	Powder + Ethanol	Greenish Brown	Green	Blackish Brown

Table 15: Fluorescence analysis of various extracts of the leaves of *Operculina turpethum*

S.NO	EXTRACTS	DAY LIGHT	UV LIGHT	
			SHORT 254nm	LONG 365nm
1	Hexane	Pale green	Yellowish green	Pale green
2	Ethyl Acetate	Greenish black	Dark green	Greenish black
3	Ethanol	Dark green	Green	Green
4	Aqueous	Brown	Pale brown	Pale yellow

There was no characteristic fluorescence seen with either the powder or the extracts.

Quantitative estimation of phytoconstituents

The phytoconstituents like glycosides, flavonoids and phenols were estimated quantitatively tabulated in Tables 16, 17 and 18.

1. GLYCOSIDE ESTIMATION

The estimation of glycoside was carried out by the general method.

Table 16: Total Glycoside content of *Operculina turpethum*

S.NO	WEIGHT OF LEAF POWDER	RESULTS
1	10 g	8.54% W/W

The percentage yield of Glycoside present in 10 g of leaf powder of *Operculina turpethum* was found to be 8.54% w/w.

2. FLAVONOID ESTIMATION

The estimation of Flavonoids was carried out by the colorimetric method.

Table 17: Total Flavonoid content of *Operculina turpethum*

Quercetin	
Concentration (µg/ml)	**Absorbance**
10	0.16
20	0.30
30	0.38
40	0.44
50	0.52
Ethyl acetate	0.39
Ethanol	0.42

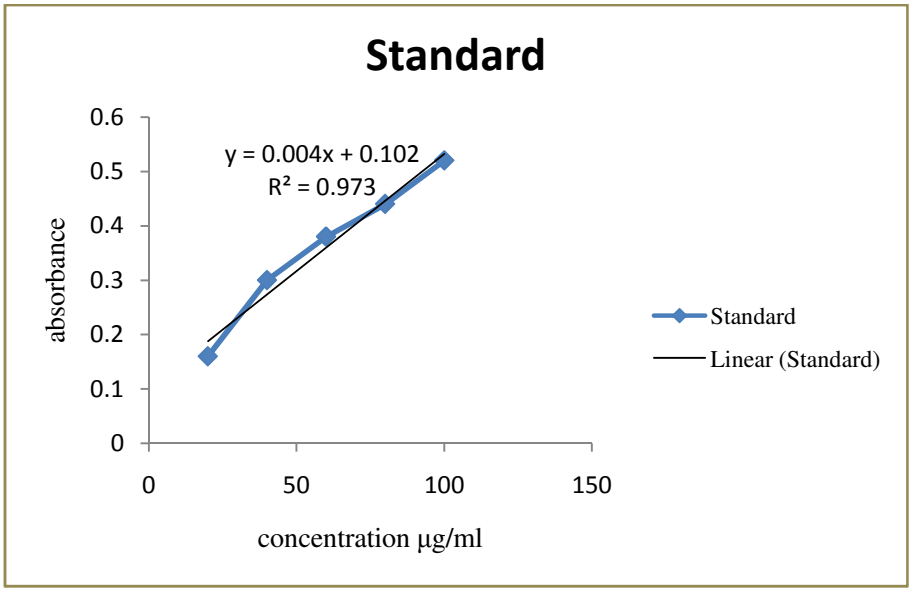


Fig. 19: Calibration curve for the standard Quercetin

The concentration of Flavonoid present in Ethanol extract was found to be 79.5µg equivalent to Quercetin in 1 mg (8%).

3. PHENOL ESTIMATION

The estimation of Phenols was carried out by the colorimetric method.

Table 18: Total Phenolic content of *Operculina turpethum*

Quercetin	
Concentration (µg/ml)	**Absorbance**
2	0.06
4	0.09
6	0.11
8	0.16
10	0.23
Ethyl acetate	0.12
Ethanol	0.18

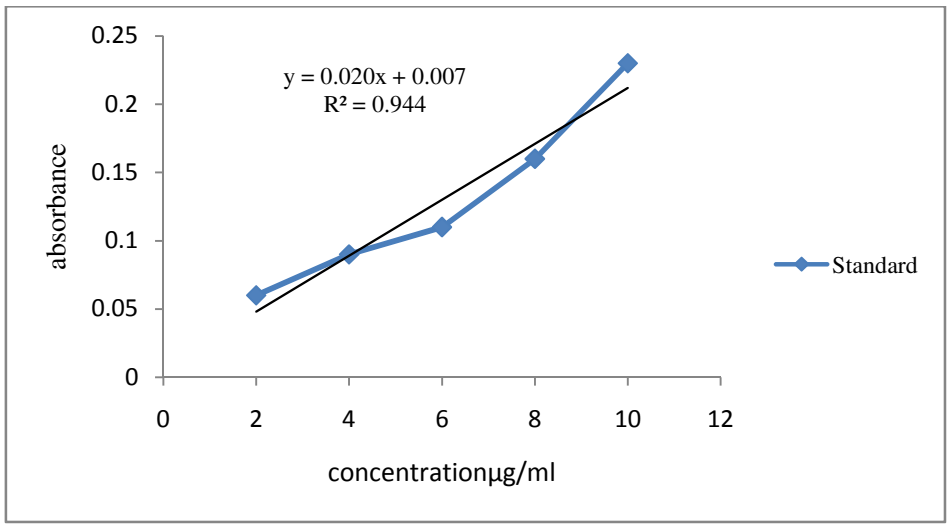


Fig. 20 : Calibration curve for the standard Gallic acid

The concentration of Phenols present in Ethanol extract was found to be 8.65µg equivqlent to Gallic acid in 1 mg (0.87%).

DISCUSSION

The phytochemical studies reveal that the leaves of *Operculina turpethum* are rich in several phytoconstituents. The ethanol extract showed the presence of all these phytoconstituents indicating the possibility, that this extract may possess all the activities of the plant.

SELECTION OF ACTIVE EXTRACT BY *IN VITRO* STUDIES

The four extracts were subjected to *in vitro* antioxidant studies and pancreatic lipase inhibitory activity and based on the results, the extract to be taken up for further studies was chosen.

***In vitro* antioxidant activity**

1. HYDROGEN PEROXIDE SCAVENGING ASSAY

The hexane, ethyl acetate, ethanol and aqueous extracts were subjected to Hydrogen peroxide scavenging assay using Ascorbic acid as standard. The results are given in Table- 19.

Table 19: Hydrogen peroxide scavenging assay

Concentration (µg/ml)	Percentage inhibition				
	Ascorbic acid	Hexane	Ethyl acetate	Ethanol	Aqueous
10	13.85	11.03	12.24	13.62	11.96
20	26.79	22.18	23.37	25.58	22.63
30	48.93	45.32	46.01	47.88	45.59
40	59.21	53.17	55.09	58.03	54.37
50	71.30	56.28	64.18	69.57	58.62

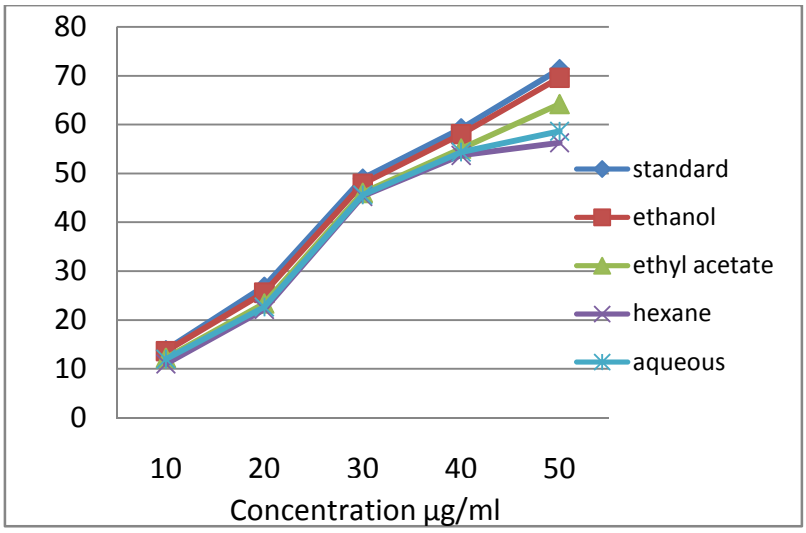


Fig. 21: Hydrogen peroxide scavenging assay

The percentage inhibition of the hydrogen peroxide scavenging assay of the Hexane, Ethyl acetate, Ethanol, Aqueous and the Standard (Vit C) was found to be 56.28, 64.18, 69.57, 58.62 and 71.30 respectively at a maximum concentration of 50 µg/ml indicating that the ethanol extract possessed maximum hydrogen peroxide scavenging activity which is comparable to the standard.

2. REDUCING POWER ABILITY ASSAY

The reducing power assay of the four extracts and the standard Ascorbic acid were carried out and the results are shown in Table- 20.

Table 20: Reducing power ability assay

Concentration (µg/ml)	Percentage Inhibition				
	Ascorbic acid	Hexane	Ethyl acetate	Ethanol	Aqueous
10	14.17	12.52	13.04	13.84	12.86
20	27.56	22.37	23.76	26.56	22.95
30	49.53	45.89	47.32	49.45	46.63
40	60.27	53.18	54.66	58.27	53.87
50	72.31	59.54	66.24	71.20	63.28

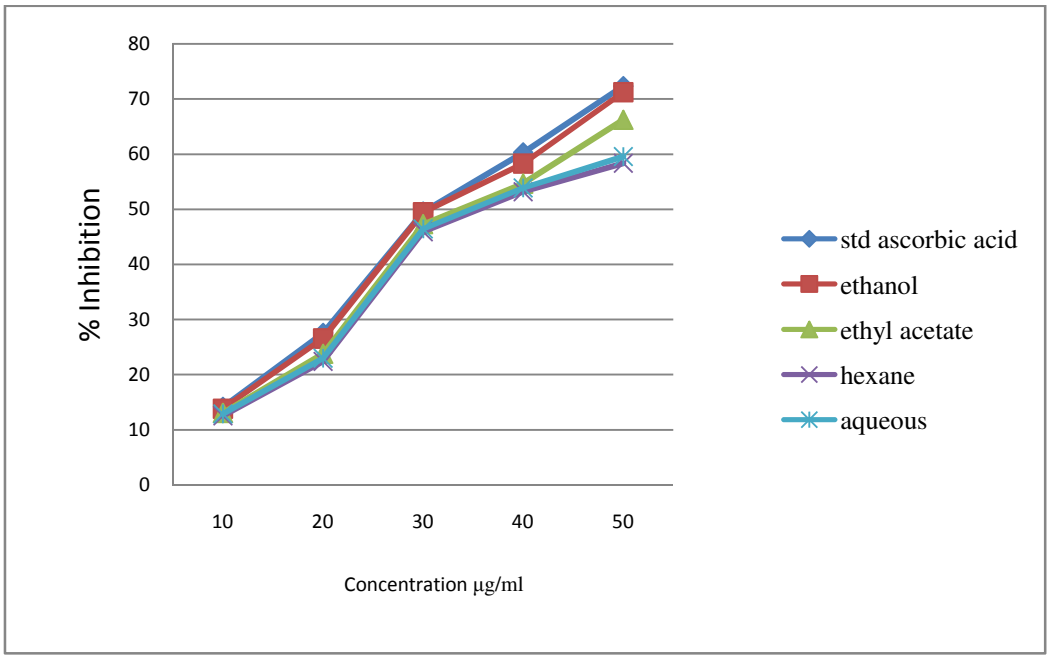


Fig. 22: Reducing power ability assay

The percentage inhibition of reducing power ability assay of the Hexane, Ethyl acetate, Ethanol, Aqueous and standard Ascorbic acid was found to be 59.54, 66.24, 71.20, 63.28 and72.31 respectively at a maximum concentration of 50 µg/ml indicating that the ethanol extract possessed maximum reducing power ability assay which is comparable to the standard.

IN VITRO INHIBITION OF CHICKEN PANCREATIC LIPASE ACTIVITY

The pancreatic lipase inhibition study was carried out using 25, 50, 75, 100µg/ml concentration for the various extracts. The results are given in Table- 21.

Table 21: Pancreatic lipase inhibition of various extracts of *Operculina turpethum*

S.no	Concentration	Anti- lipase activity				% Inhibition			
		Hexane	Ethyl acetate	Ethanol	Aqueous	Hexane	Ethyl acetate	Ethanol	Aqueous
1	25	8.8±0.2	6.5±0.1	3.8±0.4	6.7±0.5	27.86	47.98	68.85	43.76
2	50	7.9±0.3	6.2±0.3	3.1±0.3	6.5±0.5	35.24	51.30	72.95	46.72
3	75	7.2±0.2	5.1±0.3	2.4±0.3	6.2±0.1	40.98	98.19	80.32	49.18
4	100	6.8±0.5	4.5±0.4	1.8±0.4	5.7±0.3	44.26	63.11	85.24	53.27

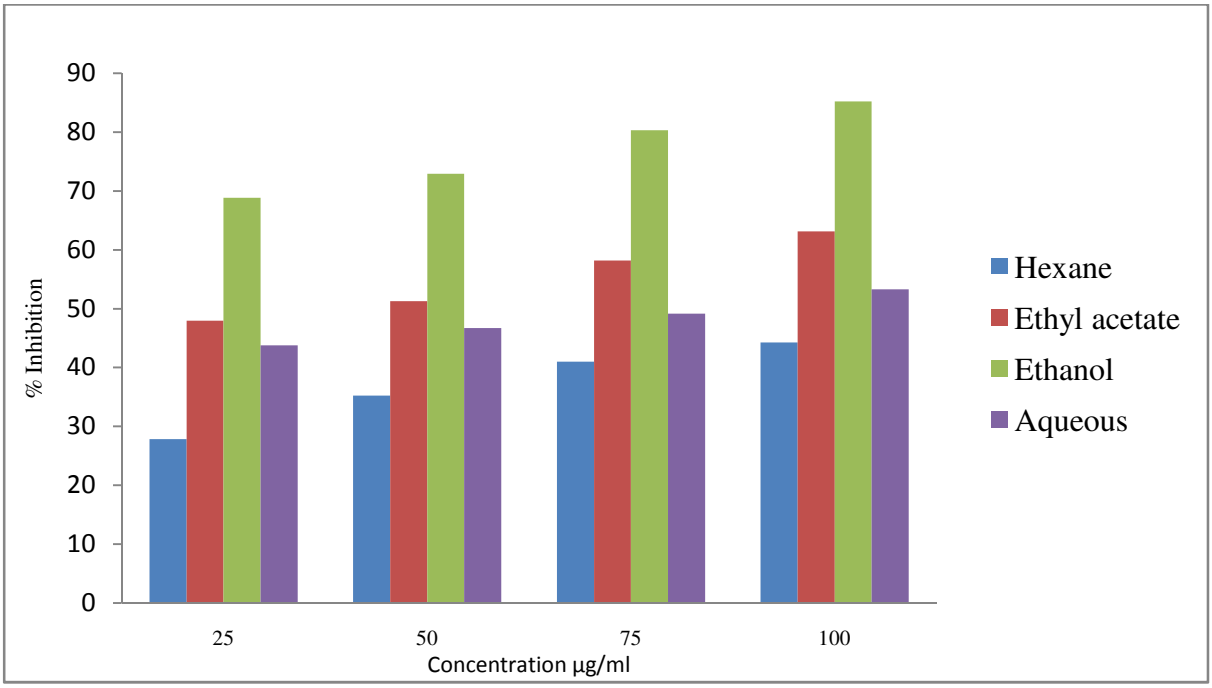


Fig. 23: Effects of various extracts on pancreatic lipase inhibition

From the study, it was found that hexane, ethyl acetate and aqueous extract showed 44.26%, 63.11% and 53.27% inhibition of lipase activity respectively. The maximum inhibition was observed in ethanolic extract which showed an inhibition of 85.24%.

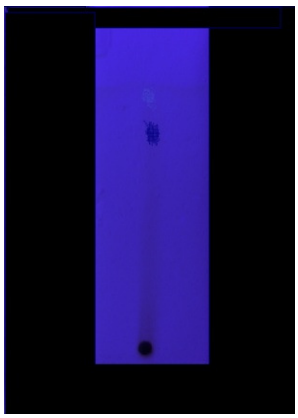
Both the antioxidant studies and lipase inhibitory activity studies indicate that of all the extracts, the ethanol extract showed the best activity. This correlates with the findings of the phytochemical study where the ethanol extract showed the presence of most of the phytoconstituents. Hence this extract was taken up for further studies.

THIN LAYER CHROMATOGRAPHY OF ETHANOLIC EXTRACT OF *Operculina turpethum*

The TLC of the ethanolic extract of leaves of *Operculina turpethum* was carried out to detect the presence of glycosides and flavonoids and the Rf values are tabulated in Table-22.

Table 22: Detection of glycosides and flavonoids

EXTRACTS	MOBILE PHASE	Rf VALUE (UV)
Ethanol	Ethyl acetate : Methanol : Water (81:11:8)	0.48
Ethanol	Toluene : Ethyl acetate : Diethylamine (70:20:10)	0.43



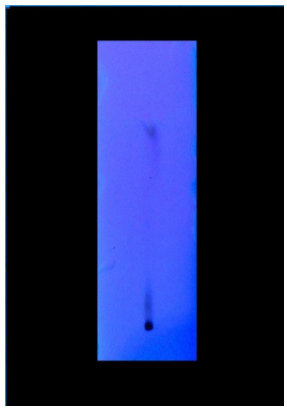


Fig. 24.Thin layer chromatography of the extract

Fig. 24.1: (Glycoside) **Fig. 24.2 :(Flavonoid)**

TLC studies showed that the ethanolic extract showed a single spot in both glycoside and flavonoid solvent system.

ISOLATION AND CHARACTERISATION BY COLUMN CHROMATOGRAPHY

The elutes obtained from Silica gel column chromatography of Ethanolic extract with different fractions are tabulated in Table-23.

Table 23: Column Chromatography of Ethanolic Extract

S.NO	ELUENT	SOLVENT RATIO	FRACTIONS
1	Hexane	100	Fraction - 1
2	Hexane : Chloroform	90:10	Fraction – 2
3	Hexane : Chloroform	80:20	Fraction – 3
4	Hexane : Chloroform	70:30	Fraction – 4
5	Hexane : Chloroform	60:40	Fraction – 5
6	Hexane : Chloroform	50:50	Fraction – 6
7	Hexane : Chloroform	40:60	Fraction – 7
8	Hexane : Chloroform	30:70	Fraction – 8
9	Hexane : Chloroform	20:80	Fraction – 9
10	Hexane : Chloroform	10:90	Fraction – 10
11	Chloroform	100	Fraction - 11
12	Chloroform : Ethyl Acetate	90:10	Fraction – 12
13	Chloroform : Ethyl Acetate	8020	Fraction – 13
14	Chloroform : Ethyl Acetate	70:30	Fraction – 14
15	Chloroform : Ethyl Acetate	60:40	Fraction – 15
16	Chloroform : Ethyl Acetate	50:50	Fraction – 16
17	Chloroform : Ethyl Acetate	40:60	Fraction – 17
18	Chloroform : Ethyl Acetate	30:70	Fraction – 18
19	Chloroform : Ethyl Acetate	20:80	Fraction – 19
20	Chloroform : Ethyl Acetate	10:90	Fraction – 20
21	Ethyl Acetate	100	Fraction – 21
22	Ethyl Acetate : Ethanol	90:10	Fraction – 22
23	Ethyl Acetate : Ethanol	80:20	Fraction – 23
24	Ethyl Acetate : Ethanol	70:30	Fraction – 24
25	Ethyl Acetate : Ethanol	60:40	Fraction – 25
26	Ethyl Acetate : Ethanol	50:50	Fraction – 26
27	Ethyl Acetate : Ethanol	40:60	Fraction – 27
28	Ethyl Acetate : Ethanol	30:70	Fraction – 28
29	Ethyl Acetate : Ethanol	20:80	Fraction – 29
30	Ethyl Acetate : Ethanol	10:90	Fraction – 30
31	Ethanol	100	Fraction – 31

The fractions obtained with same Rf value was tabulated in Table- 24.

Table 24: Thin layer chromatography of isolated fractions

Solvent system: Ethyl acetate : Methanol : Water (81:11:8)

S.NO	FRACTIONS	COMPOUND	Rf VALUE
1	Fraction – 1	-	-
2	Fraction – 2	-	-
3	Fraction – 3	-	-
4	Fraction – 4	-	-
5	Fraction – 5	-	-
6	Fraction – 6	-	-
7	Fraction – 7	-	-
8	Fraction – 8	-	-
9	Fraction – 9	-	-
10	Fraction – 10	-	-
11	Fraction – 11	-	-
12	Fraction – 12	-	-
13	Fraction – 13	-	-
14	Fraction – 14	-	-
15	Fraction – 15	-	-
16	Fraction – 16	-	-
17	Fraction – 17	-	-
18	Fraction – 18	-	-
19	Fraction – 19	-	-
20	Fraction – 20	-	-
21	Fraction – 21	-	-
22	Fraction – 22	Compound-1	0.48
23	Fraction – 23	Compound-1	0.48
24	Fraction – 24	Compound-1	0.48
25	Fraction – 25	Compound-1	0.48
26	Fraction – 26	Compound-1	0.48
27	Fraction – 27	Compound-1	0.48
28	Fraction – 28	Compound-1	0.48
29	Fraction – 29	Compound-1	0.48
30	Fraction – 30	Compound-1	0.48
31	Fraction – 31	Compound-1	0.48

The fractions 22-31 which showed an Rf value of 0.48 were pooled. The solvent was evaporated and the residue was subjected to several spectral analyses to help and identify the nature of the compound.

SPECTRAL STUDIES

The spectral studies of the compound isolated from the ethanol extract of the leaves of *Operculina turpethum* are as follows:

UV spectroscopy

UVλ_{max} – Methanol 213nm

UV SPECTRUM

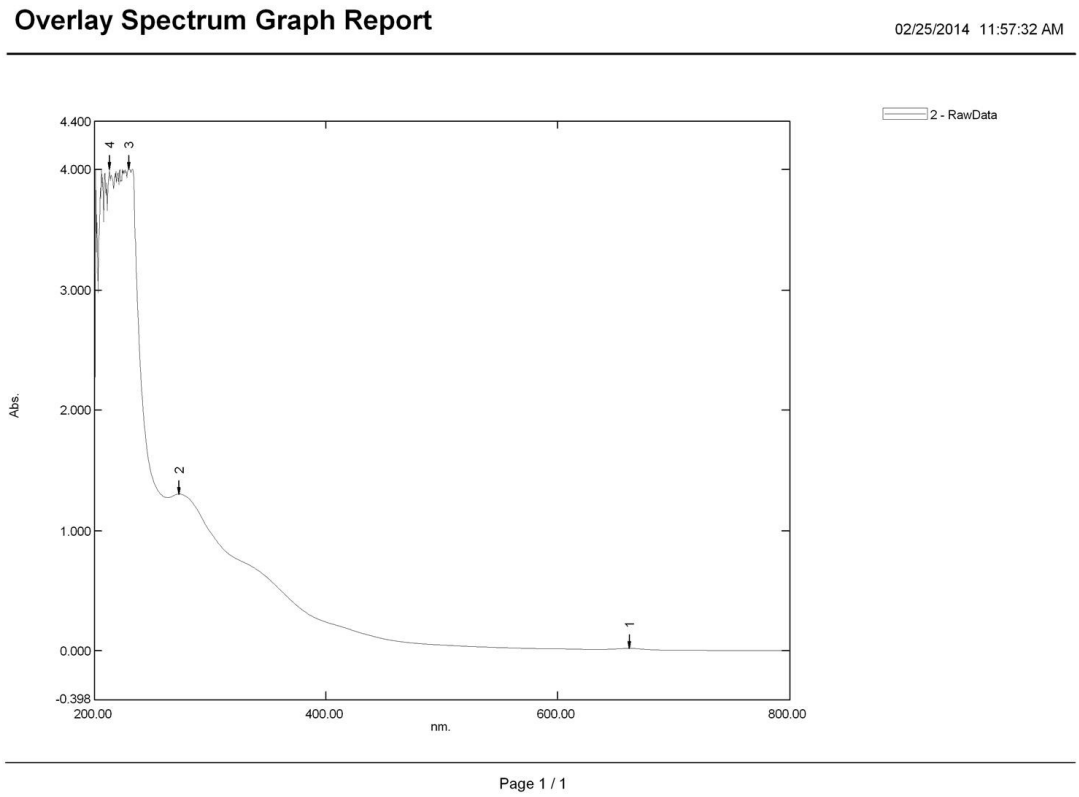


Fig. 25: UV spectra

IR spectroscopy

IR *V*cm-^{1}: Nujol mull

The functional group and type of observed wave number of IR spectra was tabulated in Table-25.

IR SPECTRUM

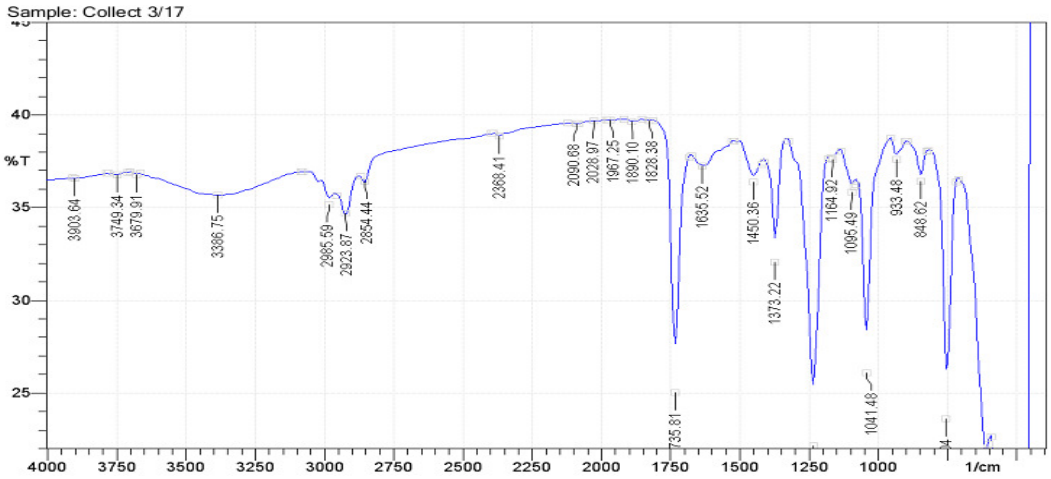


Fig. 26: IR spectra

Table 25 : IR Spectra

Assigned functional group	Observation	Observed wave number (cm^{-1})
CH_3 (s) stretching	Methyl symmetrical stretching (C-H)	2854.44
CH_3 (s) stretching	Methyl assymetrical stretching (C-H)	2985.59
CH_3 (b) bending	Methyl assymetrical bending (C-H)	1373.22
CH_2 (s) stretching	Methylene symmetrical stretching (C-H)	2923.87
C=C-C (s) stretching	Aromatic ring stretching	1450.36
C-O-C (s) stretching	Alkyl substituted ether stretching (C-O)	1164.92
C-O (s) stretching	Primary alcohol stretching (C-O)	1041.48
C-O (s) stretching	Secondary alcohol stretching (C-O)	1095.49
C-O (s) stretching	Phenol stretching (C-O)	1200.00
O-H (s) stretching	Hydroxy group stretching (H-OH)	3379.75
O-H (s) stretching	Primary alcohol stretching (OH)	3679.91
O-H (s) bending	Alcohol out of plane bending (OH)	704.00

NMR spectroscopy

^{1}H NMR SPECTRUM

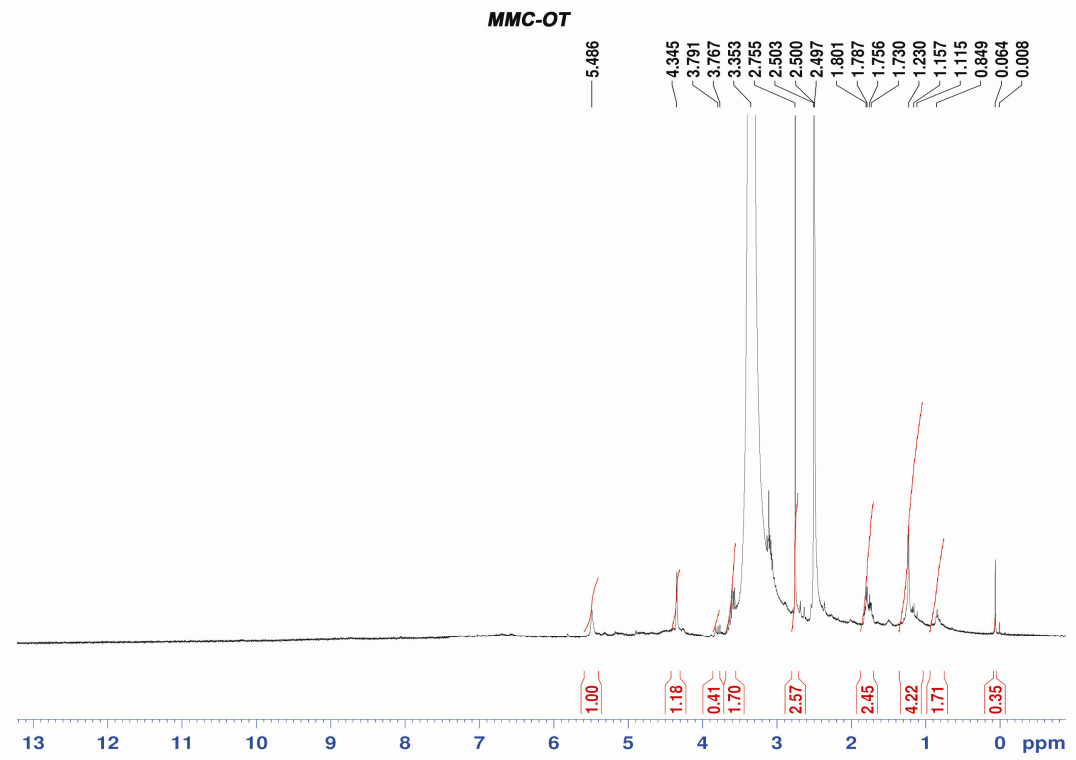


Fig. 27 : ^{1}H NMR spectra

^{1}H NMR (δ) 1.7 - Allylic methyl group

3.7 - OH group

4.3 - Aromatic group

^{13}C NMR SPECTRUM

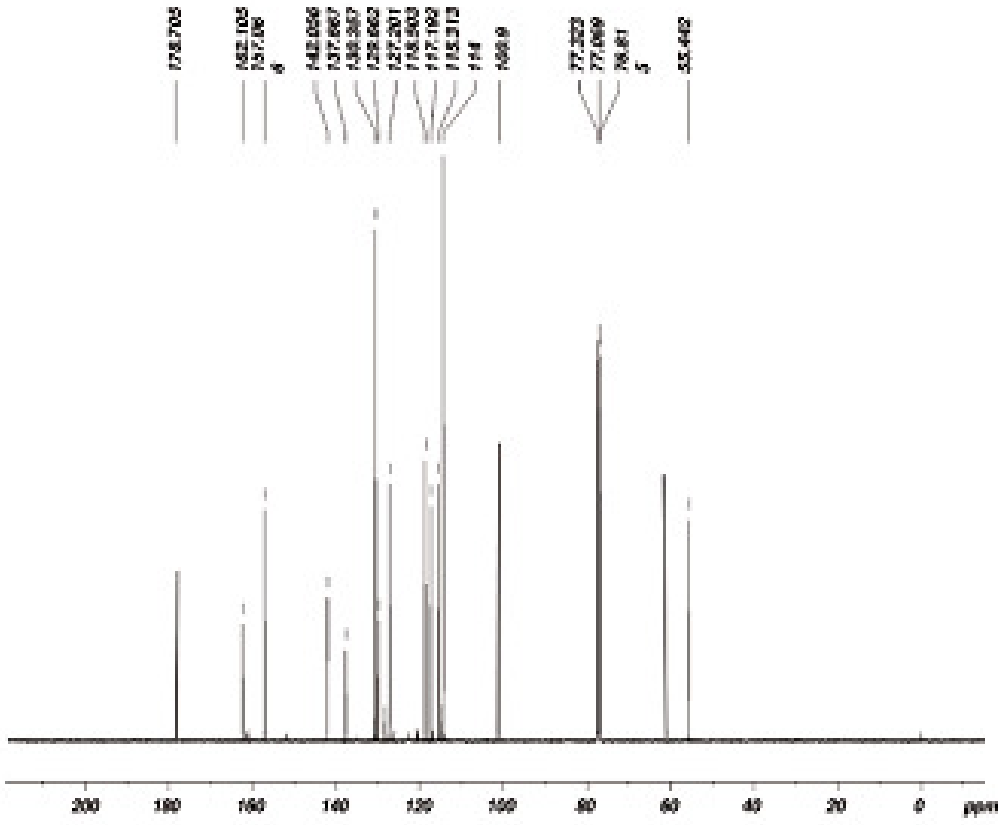


Fig . 28: ^{13}C NMR spectra

^{13}C NMR (δ)	55.4	-Tetrahydropyran
	76.81	-Tetrahydropyran
	114	- Ethylene carbon
	178.7	- Lactone carbon

Mass spectroscopy

MASS SPECTRUM

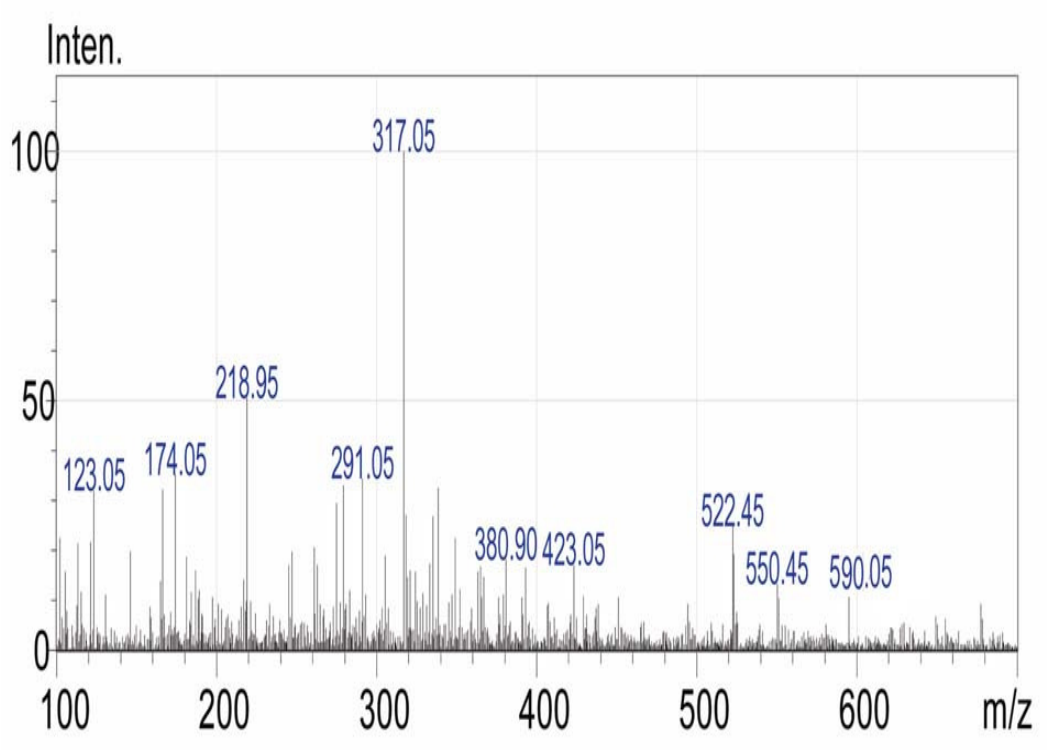


Fig. 29: Mass spectra

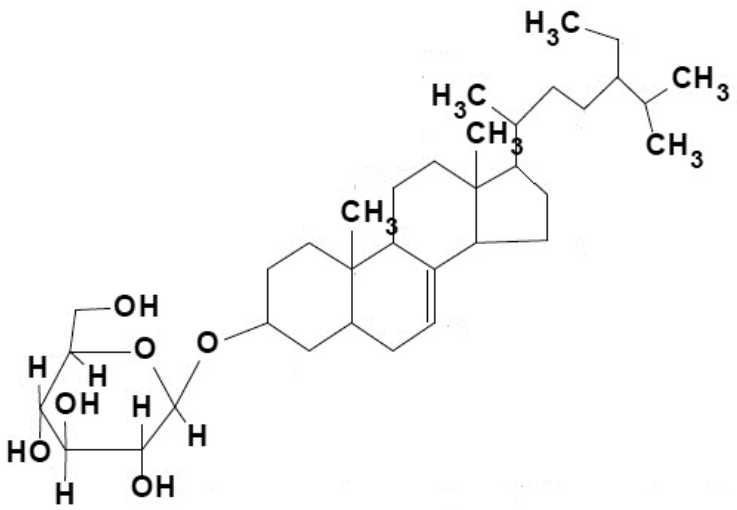
M^+ ion (Molecular peak) – 590.05

A peak characteristic at 317.05

Physical and chemical properties of the isolated compound

Molecular weight	: 590.45 g/mol
Nature	: Ash white crystalline powder
Melting point	: 284-286°C
Solubility	: Soluble in methanol, DMSO
TLC Solvent system	: Ethyl acetate : Methanol : Water (81:11:8)
Rf value	: 0.48
Detecting agent	: Visible in day light
Chemical test	: Bluish- green colour was obtained with cardiac glycoside test indicating its presence.

The molecular weight, melting point and some spectral characters matches with previously isolated compound. Hence the proposed structure may be Spinasteryl glycoside. So, further analysis may be required to carry out animal studies for isolated compound.



PHARMACOLOGICAL STUDIES

Acute toxicity studies

Ethanolic extract was found to be non-toxic when administered orally to the rats. There was no death or any toxic symptoms at the dose level of 2000mg/kg. Hence 1/10th and 1/5th of the dose (200 and 400mg/kg) were taken for the study.

***In-vivo* hypolipidemic activity**

Changes in body weight from initial to final are tabulated in Table 26.

Table 26: Change in Body Weight

Groups	Change in body weight %
Normal control	2.60
Cholesterol control	10.43
Standard control	4.86
Test group 1	7.19
Test group 2	5.65

Values are expressed as Mean ±S.E.M (n=6)

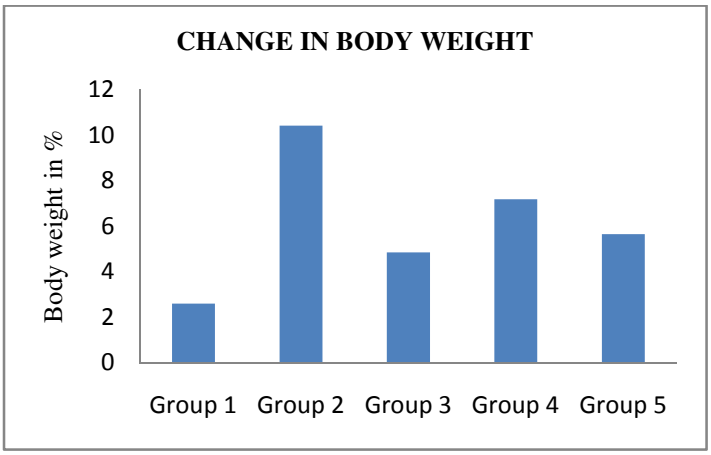


Fig. 30: Graphical representation of change in body weight

It is seen that there was a considerable increase in the body weight of animals which was treated with cholesterol alone. This increase in body weight was much reduced in animals concomitantly treated with Atorvastatin and Ethanol extract in 2 doses of 200 mg/kg and 400 mg/kg.

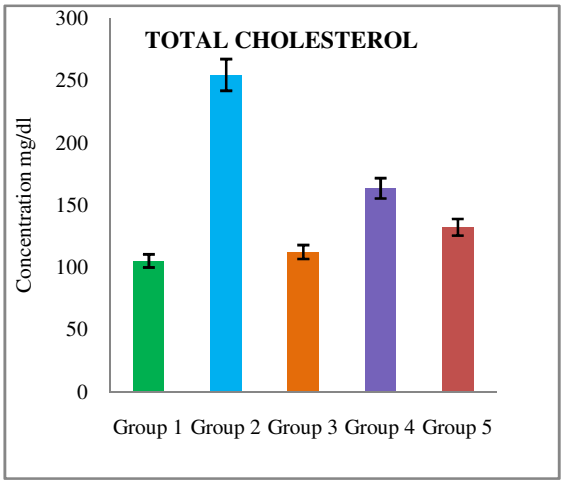
Various lipid profile parameters were evaluated for all five groups and tabulated in Table-27.

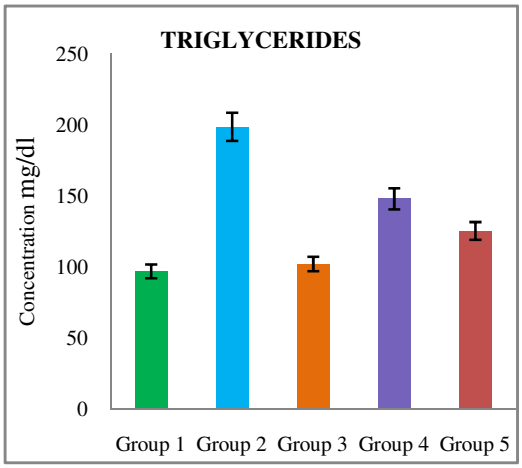
Table 27: Effect of ethanolic extract of *operculina turpethum* on lipid profile

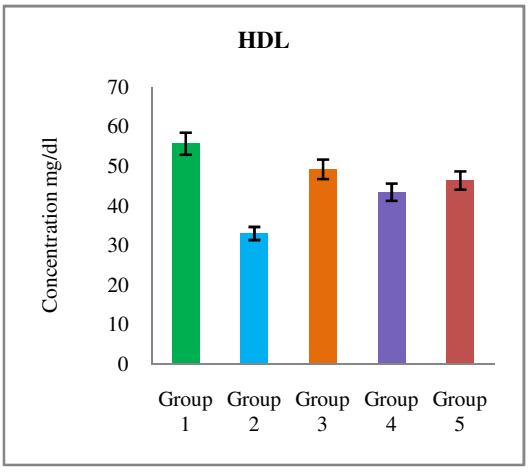
Groups	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
Group I	98.59±1.474	93.37± 0.09	54.47 ±0.896	35.52±0.079	20.33 ±0.071
Group II	246.62±0.199[a]	205.5±0.115[a]	31.70±0.126[a]	175.39±0.09[a]	37.43±0.126[a]
Group III	113.16±0.101[ab]	108.39±0.120[ab]	48.38 ±0.065[ab]	42.54±0.010[ab]	21.46±0.133[ab]
Group IV	159.06±0.175[ab]	153.46±0.118[ab]	40.50±0.123[ab]	91.55±0.146[ab]	28.43±0.087[ab]
Group V	135.04±0.170[ab]	136.39±0.106[ab]	45.27± 0.090[ab]	63.55±0.109[ab]	24.34±0.192[ab]

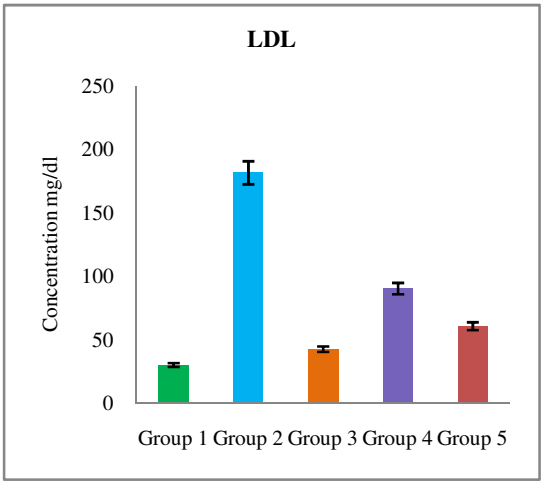
Values are expressed as Mean±SEM.
Data were analysed by one way ANOVA followed by Dunnett's t-test.
'a' values were significantly different from normal control at P<0.01.
'b' values were significantly different from disease control at P<0.01.

It is seen that in the cholesterol treated animals, the lipid values were significantly higher than the control animals. Treatment with atorvastatin significantly reduced the lipid levels. Treatment with the ethanolic extract, at both doses also significantly reduced the lipid levels and increased the HDL level. At the higher dose of 400 mg/kg the protection offered was better. However, the reduced values were still significantly different from that of the control animals.









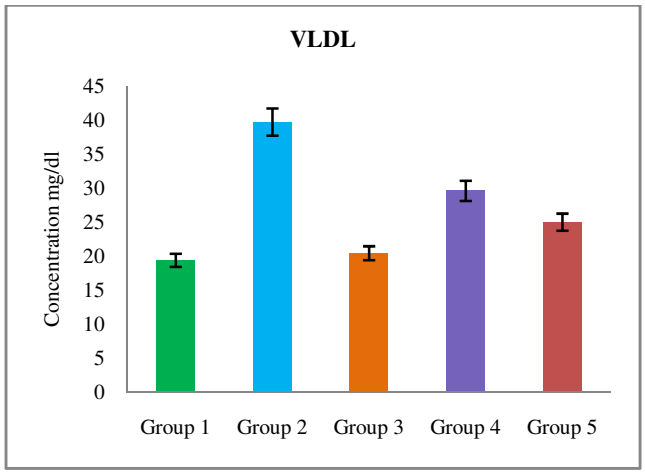


Fig. 31: Graphical representation of lipid profile

Values of atherogenic index are evaluated and tabulated in Table-28.

Table 28: Effect of ethanolic extract of *operculina turpethum* on atherogenic index and LDL/HDL

Groups	AI	LDL/HDL
Group I	0.80± 0.645	0.61 ±0.088
Group II	6.77±0.579[a]	5.53 ±0.769[a]
Group III	1.33±0.553[ab]	0.87 ±0.153[ab]
Group IV	2.92±0.412[ab]	2.26 ±1.186[ab]
Group V	1.99±0.888[ab]	1.40± 1.211[ab]

Values are expressed as Mean±SEM.
Data were analysed by one way ANOVA followed by DUNNETT'S t-test.
'a' values were significantly different from normal control at P<0.01.
'b' values were significantly different from disease control at P<0.01.

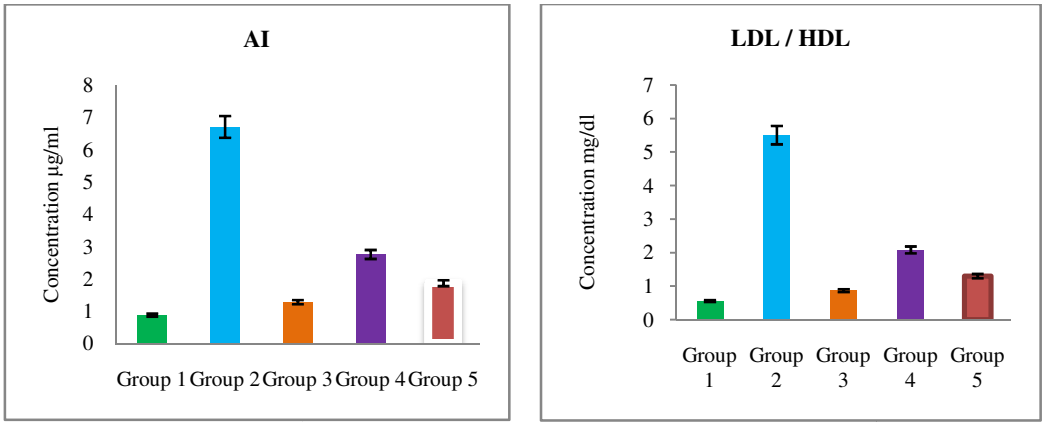


Fig. 32: Graphical representation of atherogenic index and LDL/HDL

Atherogenic index is an indicator of cardiovascular disease. The cholesterol treated group showed an increase level of atherogenic index compared to normal groups. Extract treated groups showed decrease level of atherogenic index as compared to disease group.

Liver function parameters were evaluated and listed in Table-29.

Table 29: Effect of ethanolic extract of *operculina turpethum* on liver function parameters

Groups	SGOT (U/L)	SGPT (U/L)
Group I	72.37 ±0.124	26.35 ±0.101
Group II	213.38±0.109[a]	150.40±0.064[a]
Group III	94.41± 0.073[ab]	87.55 ±0.129[ab]
Group IV	167.39±0.082[ab]	102.38±0.103[ab]
Group V	114.33±0.067[ab]	95.31± 0.084[ab]

Values are expressed as Mean±SEM.
Data were analysed by one way ANOVA followed by DUNNETT'S t-test.
'a' values were significantly different from normal control at P<0.01.
'b' values were significantly different from disease control at P<0.01.

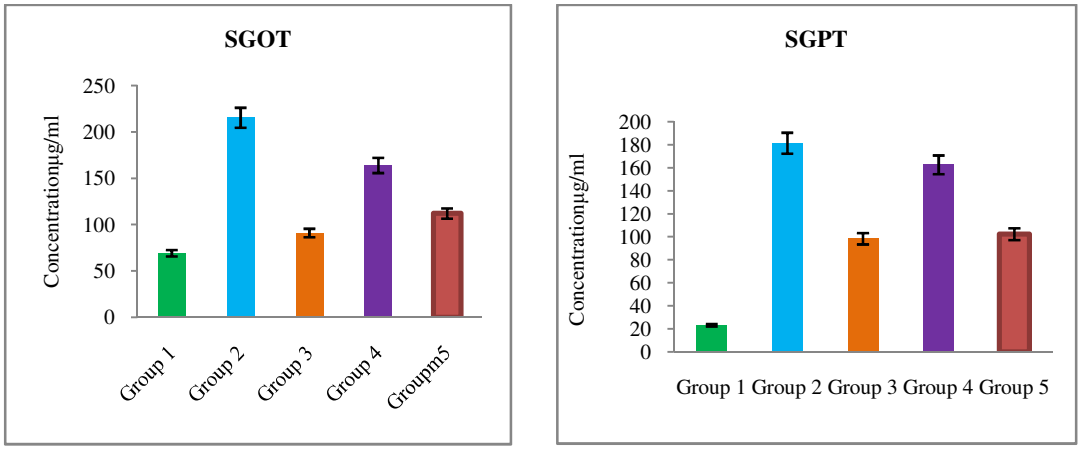


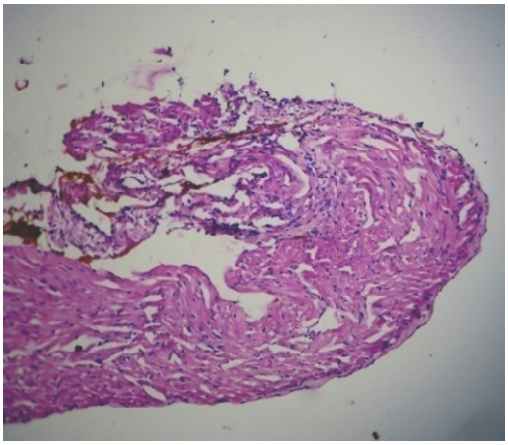
Fig. 33: Graphical representation of liver function parameters

Liver function parameters such as SGOT and SGPT also showed a significant increase in animals fed with cholesterol. These levels decreased significantly in the standard and extract treated groups.

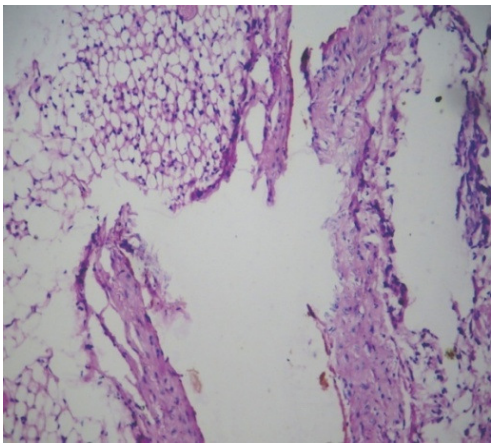
Histopathological reports are given in Fig. 34.

Histopathological studies of rat aorta

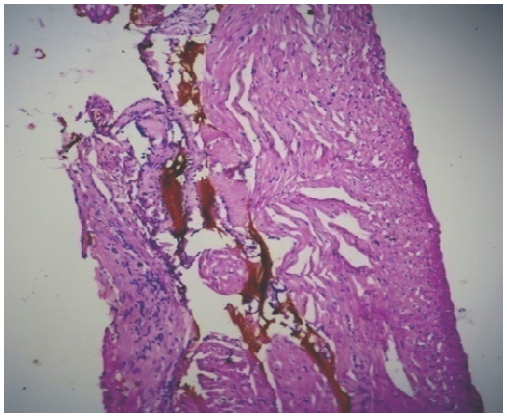
NORMAL CONTROL



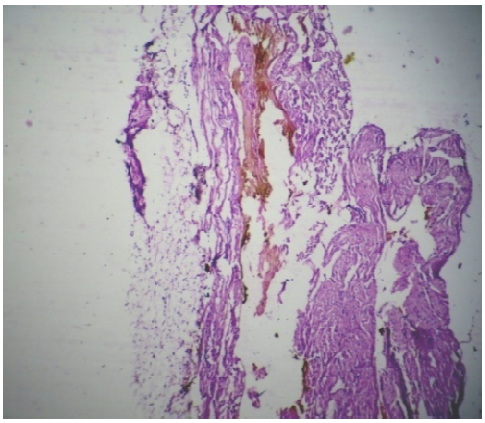
CHOLESTEROL CONTROL



STANDARD CONTROL



TEST GROUP 1



TEST GROUP 2

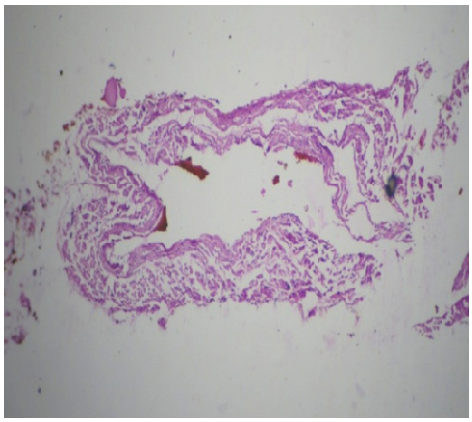


Fig. 34: Histopathology of aorta

Histopathological studies showed that the aorta section was normal in normal control group. The cholesterol treated group showed marked atheromatous thickening (plaque) in the intima and atheromatous inflammatory changes. These changes were absent in Atorvastatin treated group. Extract (200 mg/kg) treated group showed decrease in atheromatous plaque size and inflammatory changes as compared to cholesterol fed group, whereas 400mg/kg of extract treated group showed maximum level of suppression of atheromatous plaque size and atheromatous inflammatory changes.

DISCUSSION

From the pharmacological studies it was observed that the ethanol extract showed no signs of toxicity when tested at 2000 mg/kg.

The *in vivo* hypolipidemic studies showed that the ethanol extract, especially at a higher dose, improved the lipid profiles of the animals. The total cholesterol and LDL cholesterol levels were decreased whereas the HDL cholesterol level showed an increase. The atherogenic index is an indicator of cardiovascular disease. A high atherogenic index indicates a higher risk of cardiovascular disease. The atherogenic index decreased in standard group and extract treated groups. The animals which were treated with the higher dose (400mg/kg) of the extract showed a greater improvement in atherogenic index.

9. SUMMARY AND CONCLUSION

- Cardiovascular disease is one of the leading causes of death worldwide. It is a major risk for hyperlipidemia. A currently available allopathic drug for treating hyperlipidemia occurs with number of side effects and hence people are looking towards the herbal medicine. This paved a vital necessity for finding natural drugs. *Operculina turpethum* is one such plant with traditional and folklore claims which is useful in the treating Ulcer, Liver disorders, Hyperlipidemia etc.,
- The literature survey shows that very little work has been done on this plant and no hypolipidemic activity has been reported so far. Hence *Operculina turpethum* which belongs to Convolvulaceae family was chosen for the study.
- The Pharmacognostical studies on the leaves of *Operculina turpethum* was carried out first which provides unique features of the plant which may be used to differentiate it from other species. This studies reveal the following:
    - A macroscopical study indicates a characteristic feature in which leaves were ovate, oblong, cordate shaped and green in colour.
    - Anatomical studies revealed the presence of lignified and thick walled xylem cells, vascular bundle towards dorsal side and secondary phloem cells.
    - Powder analysis indicates the presence of anomocytic stomata, unicellular covering trichomes, epidermal cells and parenchyma cells.
    - Lower epidermis of the leaf showed the maximum number of stomata and epidermis.
    - The above mentioned specific characters might offer reliable clues for the correct identification of this leaf part in crude as well as fragmentary form and also ensures its differentiation from its substitutes and adulterants.
- Physicochemical studies are as follows:
    - Various physicochemical constants were evaluated for the leaves of *Operculina turpethum* such as ash values, extractive values, loss on drying, swelling index, foaming index. These values were found to be within the standard limits. This helps in confirming the identity and purity of this plant and to detect adulterants and its nature.

- Qualitative estimation of inorganic elements and heavy metals was carried out and this shows the absence of toxic metals and presence of useful elements which could be responsible for its medicinal activity.

- Phytochemical studies:
  - Successive solvent extraction was carried out to with Hexane, Ethyl acetate, Ethanol and Aqueous. The Ethanolic extract showed maximum percentage yield of 12.09% w/w.
  - The powder and extracts were subjected to preliminary phytochemical analysis. The ethyl acetate and ethanolic extracts showed the presence of alkaloids, carbohydrates, flavonoids, glycosides, phytosterols, phenols, saponins, terpenoids and tannins.
  - Fluorescence analysis was carried out to detect the fluorescent chromophore present in the powder and the extracts. No fluorescence was detected for the powder and extracts.
- *In vitro* studies :
  - The extracts were subjected to *in vitro* antioxidant and pancreatic lipase inhibitory activity. The ethanolic extract showed maximum antioxidant potential and highest pancreatic lipase inhibitory activity. Hence the ethanolic extract was taken up for further studies.
- Isolation:
  - The isolation of active phytoconstituents was carried out by column chromatography and the isolated compound was subjected to TLC and then it was interpreted by spectral analysis. The molecular weight, melting point and some spectral characters obtained was matched with previously isolated compound and it was suggested to be Spinasteryl glycoside.
- Pharmacological studies:
  - Acute toxicity was carried out in Wistar albino rats according to OECD guidelines 423. The extract showed no signs of toxicity indicating the safety of this extract.
  - The *in vivo* hypolipidemic activity was carried out by diet induced hyperlipidemia model using ethanolic extract. The studies indicate the hypolipidemic potential of the ethanolic extract which is better at the higher dose of 400 mg/kg. The histopathological studies also showed that

the atheromatous plaque size and atheromatous inflammatory changes seen in the cholesterol fed group were reversed on treatment with the extract treated group. The reversal was more marked at higher dose tested.

- Hence, from these studies it is concluded that the leaves of the plant *Operculina turpethum* possess anti-hyperlipidemic activity. Further studies on isolation of other phytoconstituents are suggested.
- Studies on the pharmacological activity of the isolated compounds will help to reveal which phytoconstituent is responsible for the hypolipidemic activity.

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